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Short communication

Simultaneous determination of levodopa, carbidopa and their metabolites in human plasma and urine samples using LC-EC

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Abstract

In this study levodopa (L-DOPA), carbidopa (C-DOPA) and their metabolites were resolved from other endogenous components present in human plasma and urine and determined quantitatively. The developed technique involved the use of a second pump, a switching valve, and a pre-column in the LC system in order to perform on-line sample clean-up and enrichment. This procedure is dependent on an effective removal of the many interfering matrix components that vitiate HPLC analysis. Several unknown endogenous electroactive compounds, present in plasma, were eliminated by the purification step, or suppressed by the pre-treatment or detection conditions. The analyses were separated on an Octyl-bonded reversed-phase column followed by amperometric detection using a carbon fibre microelectrode flow cell operated at $+0.8$ V versus silver/silver phosphate reference electrode. The cell was compatible with the mobile and the stationary phase used in the flow system without any complex surface reaction. The peak currents obtained for the different analytes were directly proportional to the analyse over the concentration range 0.02–4.0 μg ml^{-1}. Using this method, the minimum detectable concentration was estimated to be 5 and 8 ng ml^{-1} for L-DOPA and C-DOPA, respectively. Recovery studies performed on human plasma samples ranged from 93.83 to 89.76%, with a relative standard deviation of $<6\%$. The intra- and inter-assay coefficients of variation were $<7\%$. The accuracy of the assay, which was defined as the percentage difference between the mean concentration found and the theoretical (true) concentration, was 12% or better. The electrochemical pre-treatment regime described in this work permitted a longer application of the same microelectrode. The method showed a good agreement with other available methods described in the introduction and offers the advantages of being simple, less time and labour consuming, does not require additional solvents for extraction, inexpensive and suitable for routine analysis and kinetic purposes.

Keywords: Levodopa; Carbidopa; 3-orthomethyl dopa; 3-ortho carbidopa; Human plasma profiles; Urine concentration; Reversed-phase liquid chromatography; Amperometric detection; Carbon fibre flow cell

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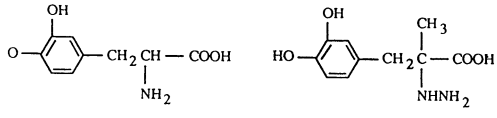
1. Introduction

Parkinsonism, first described by Parkinson in 1817, is characterised by rigidity, tremor and bradykinesia. The observation that the transmitter dopamine in basal ganglia is markedly decreased in Parkinson's disease (PD) [1] lead to the development of its metabolic precursor levodopa (L-DOPA) (3,4-di-hydroxy phenyl alanine) (Fig. 1), initially alone [2] and later combined with peripheral decarboxylase inhibitors (e.g. benserazide and carbidopa (C-DOPA)) as a substitution therapy used in combination to treat PD [3–6]. By reducing daily L-DOPA dosage requirements in this manner, the addition of C-DOPA also reduces the side effects associated with L-DOPA administration. C-DOPA (S,α-hydrazino-3,4-dihydroxy-α-methyl-benzene propionic acid; Fig. 1) is the hydrazino derivative of methyldopa, which inhibits the decarboxylation of L-DOPA in peripheral tissues and, thus, allows a greater amount of the L-DOPA dose to be transported into the central nervous system and increases its accessibility to nigrostriatal sites of action in the brain. A change in the concentration of one or both drugs in the body may influence the bioavailability and biopharmaceutic properties of the pharmaceutical preparation and subsequently their magnitude of action [7]. The treatment using these drugs provide symptomatic relief to most patients at the initial stages of disease, although unpredictable symptom fluctuations in the effect of these drugs are seen in advanced stages of the disease. The finding that fluctuations in motor performances of patients with PD receiving L-DOPA in combination with the aromatic amino acid decarboxylase inhibitors carbidopa or benserazide, are a major problem in the treatment of this disorder and a constant plasma concentration of these drugs stabilises the symptoms [7–9] have focused interest on developing different methods to monitor simultaneously the concentration of these drugs in biological fluids to assist in the management of this disease. One of the most significant advances in quantitation techniques has been the development of electrochemical detectors combined with HPLC (LC-EC), which offers the capability of selective and sensitive determination of electroactive oxidizable or reducible drug products, with little interference from biological matrix interferences [10] and with low limits of detection down to picogram quantities [11]. Few detection methods used can compare for simplicity and performance [11]. Of the numerous methods [12–29] reported for the determination of these drugs in biological fluids, few permit a simultaneous quantitative determination of both drugs and their metabolises in biological fluids. Some of these procedures are difficult to set up, expensive and require great care in maintaining satisfactory controls [18]. However, there is no general agreement as to their extraction and clean-up procedures from biological fluids [19]. Radioenzymatic assays involve conversion of DOPA to dopamine, and the intrinsic dopamine must be subtracted from the final volume [14,15]. Some of these assays proposed for clinical use were developed using biological fluids obtained from animals. Experimental animals are usually kept under much more controlled conditions than people live in. Consequently chromatograms of extracts of biological fluids from animals tend to be more reproducible and cleaner than those from people and may be misleading. HPLC methods used for the simultaneous determination of these compounds in rat brain were sensitive, but they were not selective enough to be applied to complex biological samples, such as urine and plasma, even when the samples were purified by extraction with an organic solvent [20,21], acid-washed alumina [22,24] or boric acid gel [23,28,29]. Other methods have relied upon alumina extraction for separation of cathecol compounds [24], with a separate ion-exchange isolation step for OMD and other *O*-methylated cathecol metabolites or, because of similar retention times of OMD and C-DOPA, have been unable to resolve the two in clinical



(a) L-dopa (b) Carbidopa

Fig. 1. Basic structural formulae of levodopa (a) and carbidopa (b).

samples [17,25]. The procedure using aluminium oxide, however, has several disadvantages; it is a complicated and time-consuming task, there is difficulty in activation of aluminium oxide and there is low recovery. Gas-chromatographic methods have been described [26,27], but these involve the inconvenience of multiple derivatization. In view of the importance of determining these drugs in human plasma as a tool in understanding the pharmacotherapy in PD, we present in this paper a selective and sensitive analytical procedure for the quantitation of L-DOPA, C-DOPA, and their active metabolites in human plasma using a carbon fibre microelectrode flow cell previously reported by our laboratory [30], in combination with an automated column switching assembly which included the use of two columns to extract and resolve the major drugs components and their metabolites in human plasma and urine. The use of two columns allowed the co-elution of the analytes from the first column, but are separated from the excess reagent and other side products in the sample. The peaks of interest are then switched to the second column where they are separated from each other. This particular type of column switching is often referred to as 'heart-cutting' [31]. To our knowledge such procedures have not been reported previously for the determination of these drugs and their metabolites simultaneously in biological fluids. The convenience of our method is facilitated by its rapidity, ease of sample preparation by using a fast single-extraction and enrichment step, and the use of a low cost commercially available carbon fibre. These advantages are of important consideration in performing bioavailability studies or in clinical routine analysis involving a large number of plasma samples.

2. Experimental

2.1. Chemicals and reagents

All reagents and chemicals were ACS Reagent Grade and were used without purification. Sodium dihydrogen orthophosphate was purchased from Fluka (Buchs, Switzerland). Orthophosphoric acid was supplied by BDH (BDH, Poole, Dorset, UK). All other chemicals were obtained from Sigma (Pool, Dorset, UK). Water was distilled and then further purified by passing through a Milli-Q water purification system (Millipore, Milford, MA, USA). Plasma samples were obtained from normal human volunteers. Heparinized plastic tubes used for whole blood collection 'LiHeparin Menovette', were supplied by Sarstedt (Numbrecht, Germany). Carbon fibres, 14 μm in diameter, were obtained from AVCO (Lowell, MA, USA). The surface of these fibres has no external coating. Silver epoxy was purchased from RS components (Corby, Northants, UK) and the glassy carbon used in the comparison studies was obtained from EG&G Princeton Applied Research (Princeton, NJ, USA).

2.2. HPLC-mobile phase

The mobile phase used was a mixture of 16.5 g of sodium dihydrogen orthophosphate, 980 ml of water, 1.0 ml of 0.10 M disodium ethylenediamine tetra-acetic acid (EDTA), 20 ml of methanol and 1.2 ml of 0.5 mM sodium heptanesulphonate (HAS) with the pH adjusted to 3.4 with 1 M orthophospharic acid. The mobile phase was filtered through a pore size 0.45 μm cellulose membrane filter (HVLP, Millipore, Bedford, MA, USA), degassed under reduced pressure and used at ambient temperature. With a flow-rate of 1 ml min^{-1}, the elution using this mobile phase was carried out at this temperature. The EDTA was used to sequester any oxidisable metal ions.

2.3. HPLC-columns

An Octyl-bonded/reversed phase column (12.5 cm × 4.0 mm I.D. 5-μm particle size, Lichrosorb RP-8 Hibar) was used for the purpose of the purification of deproteinized plasma and urine samples as well as for preconcentration of the different analytes. The second column, 25.0 cm × 4.0 mm I.D. 5-μm reversed-phase column (Ultrasphere), was used for the separation of the

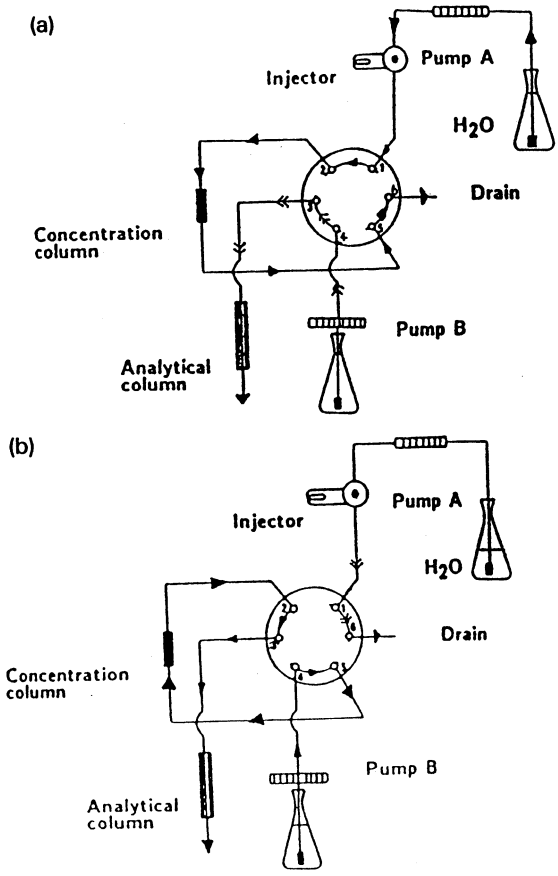


Fig. 2. Sketch of the flow pattern through the column switching system, used for retaining the analyses in the pre-concentration column (a) and eluting the drugs from the pre-concentration column (b).

compounds eluted from the concentration column. A 2 μm pre-column filter (Rheodyne) was also used. The column was normally operated at a back-pressure of 93 bar. The analytical column was primed by recycling the mobile phase overnight at a flow-rate of 0.3 ml min^{-1} At the end of a use period, the column was washed free of mobile phase components with 400–500 ml of water and then was returned by means of a gradient to 70% methanol for storage. The column in use retained good efficiency and resolution for over 6 months despite prolonged exposure to aqueous solutions, and the matrix effects of the biological samples used.

2.4. Equipment

Plasma centrifugation was performed on a Microfuge 12 microcentrifuge from Beckman Instruments (Fullerton, CA, USA). All analyses were performed on HPLC waters system consisted of two pumps (Waters Model 501). Sample introduction was via a Rheodyne (Cotate, CA, USA) Model 7125 injection valve, fitted with a 25 μl loop for direct injection. For the purpose of extraction by column switching, the injection was fitted with a 1 ml loop and a second pump (pump A), and the concentration column was connected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. Fig. 2 illustrates schematically the configuration of the column-switching system at the valve positions A and B. At position A, the loading/washing eluent delivered by pump A eluted the gross plasma interferents to waste, whereas the drugs and their metabolites were completely retained. When the valve rotated to position B, the analytes were swept onto the concentration column by the mobile phase where it was separated and subsequently detected. Oxidative amperometric measurements were performed using a Model 400 EC potentiostat (EG&G Princeton Applied Research) connected to the flow cell by crocodile clips. The resultant signals were recorded on a Philips Model PM 8261 x-t recorder (Eindhoven, The Netherlands) at a chart speed of 33 mm h^{-1}. The peak currents (measured as peak heights on the recorder) as a function of concentration were then measured for quantitative analysis.

2.5. Fabrication of micro-electrode flow cell

The micro-electrodes used were fabricated from carbon fibres, the diameter of which is 14 μm. There are two prominent features of the electrochemistry attained at these electrodes: first, their small size results in predominantly diffusion-controlled currents that are time-independent within a very short time of the initial application of potential; secondly, surface conditions can contribute significantly to the electrochemical reactions at the electrode. The method of inserting the carbon fibre into a polyethylene tube is shown in

Fig. 3(a). The needle holes were sealed by gently heating with an electric iron. The construction of the counter electrode was described previously [30]. The working, reference, and counter electrode were mounted in a T-tube arrangement (Fig. 3(b)), and the working electrode was connected to the outlet of the HPLC column (downstream of the working electrode). Hence the mobile phase eluent passed first through the working electrode and then via the counter electrode to waste. Placing the auxiliary electrode in this position reduces the uncompensated resistance to negligible value even with the low ionic strength of the mobile phase in use. With such a low uncompensated resistance the interfacial electrode potential is not significantly influenced by sample concentration, and a wide linear range was obtained.

2.6. Optimisation of detection potential

In order to determine the optimum applied potential for both drugs, hydrodynamic voltammograms were obtained by repeated injections of 40 ng ml^{-1} L-DOPA and C-DOPA standard solutions into the chromatography system and recording the peak currents, at fixed applied potentials versus Ag/Ag_3PO_4. The detector operating oxidation voltage was increased from roughly 0.2 to 1.2 V in 0.2 V increments.

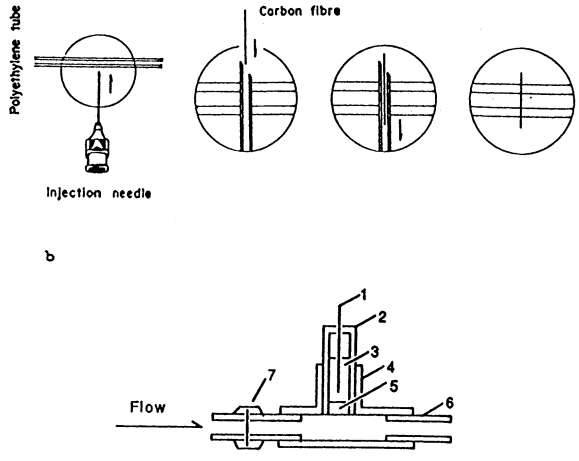


Fig. 3. Structure of the carbon fibre micro-flow cell: (a) Method of fixing a carbon fibre into polyethylene tube. (b) Diagram of the carbon fibre micro-flow cell consisting of (1) silver wire coated with Ag_3PO_4, (2) reference electrode body, (3) internal reference solution, (4) T- tube, (5) ceramic rod, (6) stainless-steel counter electrode and (7) fibre flow electrode.

2.7. Standard solutions and linearity

Plasma standards were prepared from blank human plasma spiked with L-DOPA, C-DOPA, and their metabolites (3-OMD, and 3-OMC), in the range 0.02–4 μg ml^{-1}, which is enough to cover the range of expected patient values (usually up to 3.5 μg ml^{-1}. The working dilutions were divide into aliquots, which were frozen immediately at −70°C, protected from light for maximum stability, till used for analysis. Each calibration point was run in triplicate over five consecutive days, and the observed coefficient of variation (C.V.) for each standard curve was calculated from the least-squares regression equations. The overall performance of the system was verified periodically by injection of standard samples.

2.8. Extraction recovery

The extraction efficiency and recovery of the assay was assessed at concentrations of 0.5, 1.0 and 1.5 μg ml^{-1}. Six replicates of each concentration containing the two compounds in human plasma were injected into the column and extracted according to the method described. The recovery of both drugs from plasma was assessed by comparing the peak current of the extracted plasma samples with the peak current of authentic (unextracted, aqueous) standards, which were directly injected (i.e. without column switching) onto the analytical column at the concentration levels, and the assay recovery was calculated using the following equation:

$$\% \text{ recovery} = (\text{mean peak height plasma extract}) / (\text{mean peak height standard}) \times 100$$

2.9. Specificity of the method

In order to examine the presence of any interferences from the active metabolites of both drugs and other impurities that might exist in the phar-

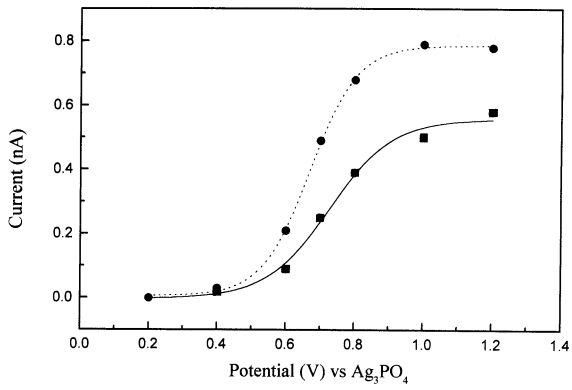


Fig. 4. Hydrodynamic voltammograms of levodopa (●) and carbidopa (■) obtained by injections of a standard solution prepared in the mobile phase into the chromatograph. Mobile phase: see text, pH 3.4; working electrode: 14 μm carbon fibre at different potentials versus Ag/Ag_3PO_4.

maceutical preparation used in the method developed. The sensitivities, retention times and extraction recoveries for these two drugs were determined by the above procedures in the presence of these substances at a concentration of 1 μg ml^{-1}.

2.10. Urine analysis

Samples with 2.0 ml of chlorhexidine gluconate added as a preservative were acidified immediately to a pH of 2 with a 6 M HCl to increase the stability [11]. The samples were then deproteinised by the addition of 0.01 M HCl and l M perchloric acid, the mixture was vortexed and centrifuged at 630 g at 5°C for 20 min [12]. Before analysis, the samples were filtered through 0.22 μm cellulose filters (Millipore, Sergrate MI, Italy).

3. Results and discussion

3.1. Optimisation of oxidation potential

Each of the analyses contains a phenol or catechol functionality, which can be easily detected by direct anodic oxidation at relatively low potential [11,32]. The detector operated in the amperometric mode with a fixed potential sufficiently positive to force the ortho-hydroxy groups to undergo loss of two electrons and two protons, yielding orthoquinone. Fig. 4 shows the hydrodynamic voltammograms of L-DOPA and C-DOPA prepared in the mobile phase. The voltammograms represent a rapidly achieved mass transport steady state. Plot of applied potential (E) against peak current exhibited one wave for each drug and showed a distinct sigmoidal dependence upon applied potential. The magnitude of the current is directly proportional to the bulk concentration at every point along the voltammetric curve. The single anodic peak obtained in the range studied verifies that both compounds underwent a single oxidation process within this range and was also dependent on the change in pH, although it is often desirable to operate an electrochemical detector on the limiting current plateau; in this case it was advantageous to operate on the rising part of the curve. The lower the potential chosen, the lower the background current produced and better sensitivity is obtained. Higher potentials resulted in greater background current and the electrochemical detector was unlikely to be useful for trace analysis as the background oxidation became exorbitant. A working potential of $+0.8$ V afforded a significant reduction in background current, which resulted in better signal-to-noise ratios and this potential was selected for the reminder of the LC-EC studies.

3.2. Separation of the analytes in human plasma

Reversed-phase chromatography appears to be most useful in the analysis of mixtures of such substances which posses both acidic and basic organic functional groups. Deproteinized plasma samples were injected directly into the HPLC system; every four samples were followed by a standard. The first step in the set-up of the switching/separation system involved the selection of a suitable pre- (or concentration) column, which would retain the analytes while other endogenous components are eluted to waste. Among the different columns used for this purpose, an Octyl-bonded/reversed phase column (12.5 cm × 4.0 mm I.D. 5 μm-particle size, Lichrosorb RP-8 Hibar) showed the most favourable retention characteristics for both drugs and their metabolites. The next

stage was to find two compatible eluents of different elutropic strengths; one should have a poor/elution capability to concentrate the analyses on the pre-column, and a second strongly eluting solvent to elute the analytes off the pre-column and onto the analytical column. The washing eluent should also be miscible with the mobile phase, as even slight incompatibility could result in a slug of solvent travelling down the analytical column, partially carrying sample components which may cause band broadening. The use of a 0.05 M solution of perchloric acid allowed the purification of plasma samples by washing out neutral and acidic compounds while the analyses (containing amino groups) were retained by the pre-column. A wash time (defined as the length of time between injection and switching of the valve) of 1.5 min was found to be satisfactory to provide a good clean-up of the plasma components without causing the analytes to elute. Six replicate injections of plasma samples containing the different analyses yielded a relative standard deviation (RSD) $> 5\%$ for each substance. The configuration of the LC-EC system, the size of the packing materials used in both columns and the choice of the suitable columns and mobile/washing eluents had reduced the back-pressure and pressure jumps caused by switching of two HPLC columns, and in return reduced the possibility of unacceptable baseline disturbances or any peak broadening usually caused by the use of large particles. Memory effects, a usual problem occurring when using column switching chromatography, was never encountered using such a configuration.

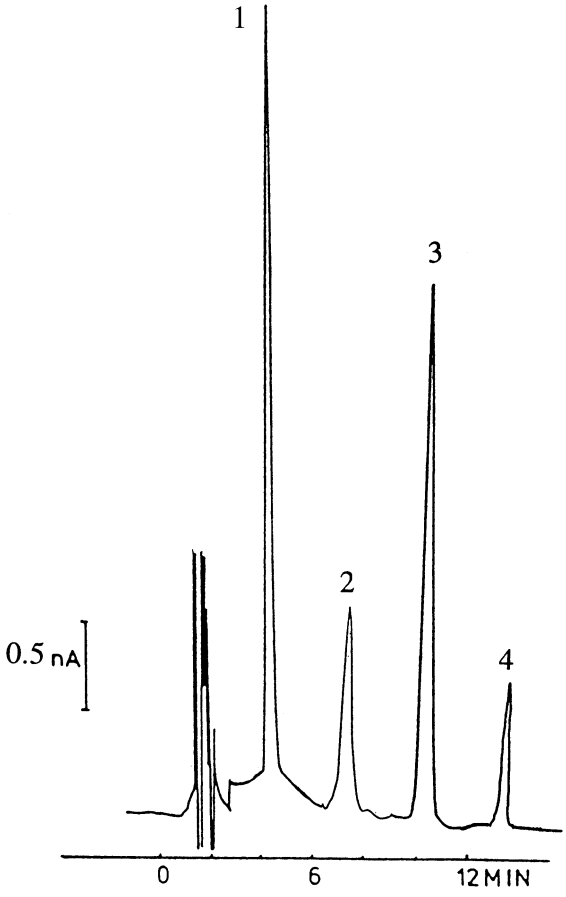


Fig. 5. A chromatogram obtained by direct injection of deproteinized plasma sample containing L-DOPA, 3-OMD, C-DOPA and 3-OMC. Peaks: 1 = L-DOPA; 2 = 3-OMD; 3 = C-DOPA; 4 = 3-OMC.

Fig. 5 shows the chromatogram of a spiked plasma sample containing 0.3 μg ml^{-1} L-DOPA/3-OMD and 0.35 pg ml^{-1} C-DOPA/3-OMC. The figure clearly indicates that under the experimental conditions, both drugs were well resolved from the endogenous plasma peaks flanking it and their main metabolites. The mobile phase was optimised by careful examination of all major variables, including pH and ionic strength, to determine their influence on enhancing peak separation without prolonging the analysis time for each sample. In such separations, an acidic pH proves optimal and modest pH adjustments can produce significant changes in the retention time characteristics of these substances. The pH selected for the mobile phase did not significantly alter the peak characteristics, even after 6 months of continuous use. There was no interference from the heparin and sodium metabisulphite, which are added to the blood as anticoagulant and antioxidant, respectively. The retention sequence for the different analyses can be explained due to the basis of the difference in the polarity. The primary amine levodopa elutes well before the less polar hydrazine-containing carbidopa. Methyl dopa and methylcarbidopa, due to the extra methyl group, are retained more, relative to L-DOPA and C-DOPA, respectively. The importance of separating and detecting these metabolites lies in the side

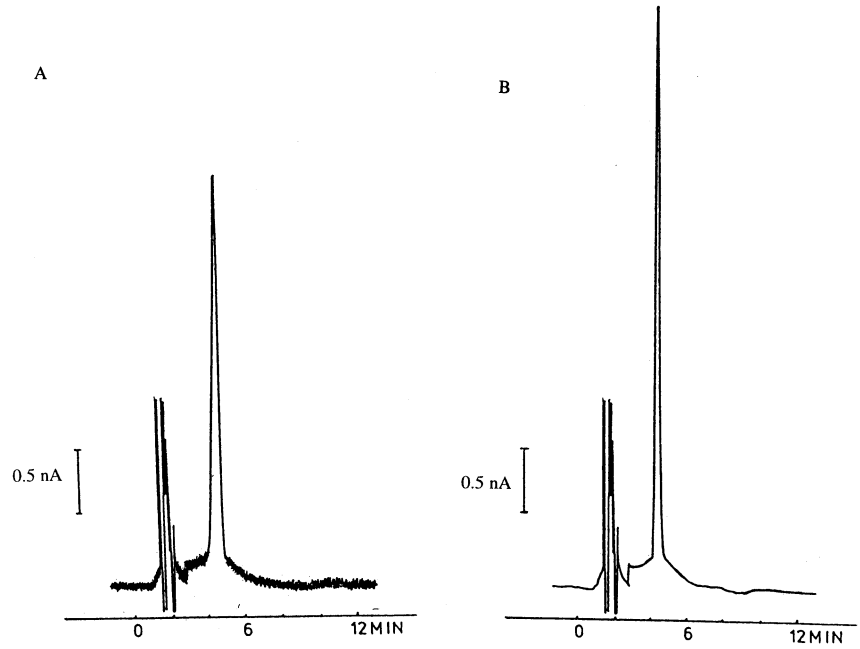


Fig. 6. Chromatograms of 0.3 μg ml^{-1} levodopa obtained by analysing the drug (A) before and (B) after electrochemical pre-treatment of the working electrode.

effects caused by their presence during long term therapies. Side-effects (dyskinesia, on-off phenomena and psychoses) is attributed to the high levels of 3-OMD in plasma and/or the plasma 3-OMD/L-DOPA ratio [33,34]. Furthermore, hypokinesia (a situation associated with low plasma levels of L-DOPA) can occur due to a competition for transport across the blood–brain barrier between L-DOPA and 3-OMD [35], and the determination of *O*-methylated products might be of clinical significance in psychiatric disorders [36]. Other metabolic pathways are unimportant [37].

3.3. Pre-treatment of the micro-electrode

Due to its inherent surface characteristics, carbon often exhibits poor electron-transfer kinetics. This seems to be especially true for analysis of anionic species. Since the voltammetric response at carbon surfaces often degrades at an enhanced rate when placed in biological media, presumably due to adsorption of large molecules to the electrode surface, a process is required to renew the carbon surface before obtaining electrochemical information. The surface of the electrode used often changed after several runs (Fig. 6(a)). One reason for such changes is due to either the adsorption of species from solution or chemical changes which could have occurred on the electrode surface itself possibly because of the different reactions between the analyte and the electrode surface or due to a resultant by-product. These changes produced a high baseline noise resulted in variations in sensitivity, reproducibility and selectivity. A variety of methods have been used in the past by different workers, among these was laser treatment of the working electrode [38] and electrochemical pre-treatment [39].

The latter was preferred due to the configuration of the system and the availability of such treatment within the system used. Anodisation of the microelectrode at $+1.0$ V for 60 s, then cathodisation at -1.0 V for another minute, followed by equilibration at the working potential for 5 min was enough to remove any surface contaminants or inhibitory layers which hindered transport of electrons and oxidation of the species at the electrode surface. The activation of the

electrode surface as a result of the pre-treatment process can produce quinoidal functionalities and improve the current response [40]. Fig. 6b illustrates clearly the influence of the pre-treatment regime in the base-line noise and the signal-to-noise ratio level.

3.4. Method evaluation

3.4.1. Linearity and detection limits

Linear calibration graphs of peak heights of the standards, versus the concentration of the standards were constructed by analysing spiked blank plasma over five days in the concentration range 0.02–4.0 μg ml^{-1}. This concentration range is within the therapeutic levels for these drugs [7,41–43]. The resulting current was directly proportional to the concentration of the oxidisable species. The plotted points represented the means of at least five replicate injections per standard. The curves showed a good linearity between the detector response and the analyte concentration. For each of the five regression lines the correlation coefficients (r^2) were all greater than 0.993. The coefficient of variation of the slopes ($n = 5$) of the regression lines calculated from the least squares regression equations, ranged from 3.0–8.74%, with intercepts all close to zero.

The sensitivity of the method is often expressed as the limit of detection (LOD). The LOD for the analysis is defined as the standard deviation of the base line noise. The measurement of the base line noise is not ambiguous and a more useful way to describe the sensitivity when blood or urine samples are analysed could be to calculate the instrumental limit of detection (ILOD) and the limit of quantitation (LOQ). The ILOD is the amount of an analyse giving a peak height three times the standard deviation of the base line noise without any matrix interference, whereas the LOQ is the concentration of an analyse in the matrix that could be determined with a precision (a signal-to-noise ratio of 3) under the given analytical procedure. In our case the ILOD for L-DOPA and C-DOPA was found to be 5 and 8 ng ml^{-1}, respectively. The LOQ was ca. 8 ng ml^{-1} for L-DOPA and 10 ng ml^{-1} for C-DOPA. This limit of quantitation is adequate for clinical analysis and pharmacotherapeutic studies and comparable to those values obtained by previous workers [3,6,44–46]. The use of relatively low potential, incorporation of reference and auxiliary electrodes in the same cell and recycling of the mobile phase, all resulted in reduction of the background noise and improved LOD and LOQ values.

3.5. Precision and extraction recovery

The column switching technique usually gives better precision than the traditional direct injection technique for micro-samples [47]. The precision of the method based on peak-height measurements was evaluated by replicate injection exhibited a coefficient of variation of $< 6.0\%$. Peak-height measurements were found to yield the same reproducible results as peak-area determinations, and there was no need to include an internal standard in the analysis due to the good recovery and reproducibility obtained. The mean absolute recovery achieved with the described extraction procedure was always greater than 91.0% for L-DOPA and 89% for C-DOPA. Table 1 illustrates the precision and extraction recovery for both drugs using the technique described.

3.6. Selectivity of the method

The possible potential various foreign compounds were studied; these compounds were selected according to (a) compounds which can be present as impurities in combination with the drugs in different pharmaceutical formulations and affect the analytical process and (b) other drugs that might be administered with these drugs. United States Pharmacopoeia (USP) [48] specifies methyl dopa, 3-methoxy tyrosine, and 3-*O*-methyl carbidopa as impurities in the analysis of L-DOPA–C-DOPA combination formulations. Interference from some of these substances in the determination of the drugs was tested in two different studies using the proposed technique. The separation peak height, retention times and the recovery of the analyses were not affected by the presence of these substances. Errors $< 2\%$ were calculated by comparing the results with the

Table 1
Precision and extraction recovery

Compound	Concentration added (μg ml^{-1})	Mean concentration found (μg ml^{-1})	Recovery (%)	Coefficient[a] of variation (%)	Accuracy[b]
L-DOPA	0.5	0.459	91.83	4.70	4.10
	1.0	0.925	92.50	3.92	7.50
	1.5*	1.407	93.86	3.10	9.30
C-DOPA	0.5	0.449	89.80	5.20	5.10
	1.0	0.913	91.31	4.63	8.70
	1.5*	1.387	92.47	3.75	11.30

[a] Mean of six samples from single plasma pool assayed in triplicate.
[b] The percentage difference between the mean concentration found and the theoretical concentration.
* Highest concentrations observed in clinical samples.

corresponding results obtained with a solution containing only both drugs. Other drugs such as tricyclic antidepressants (Desipramine) that might be co-administered with both drugs was not observed under the experimental conditions. The selectivity and the resolution of the column was enhanced by the use of an ion pairing species (HSA), and by the admixture of an organic modifier such as methanol in the mobile phase.

3.7. Inter- and intra-assay variability

Within-day variabilities were established for five different days concentrations (0.25, 0.5 and l.5 μg ml^{-1}) for dopa and carbidopa by adding the drugs to blank plasma over five consecutive days. Peak current values were interpolated on the individual regression lines to yield three new values of amount found at each concentration level for every day. Between-day variability was then calculated by obtaining the mean C.V (%). Results from this experiment are presented in Table 2, and they show that the overall assay variations at 0.25, 0.5 and l.5 μg nl^{-1} were $< 7\%$ for both drugs.

3.8. Stability studies

The stability of the electrode is of importance in order to define the period of time over which the electrode can be used without a significant decrease in the sensitivity. After 1 week it was observed that a significant decrease in the sensitivity has occurred even with the use of the electrochemical pre-treatment procedure. The electrode response was at 45% of its initial value, with relative standard deviations greater than 13%. The amperometric detector was left on as long as

Table 2
Inter- and intra-assay variability

Compound	Inter-assay		Intra-assay	
	Concentration[a] (μg ml^{-1})	C.V. (%)	Concentration (μg ml^{-1})	C.V. (%)
Levodopa	0.25	5.1	0.25	6.73
	0.50	4.70	0.50	5.91
	1.50	3.56	1.50	4.54
Carbidopa	0.25	5.34	0.25	6.86
	0.50	4.55	0.50	5.34
	1.50	3.21	1.50	4.23

[a] Individual values are the mean of three measurements.

Table 3
Absolute recovery of L-DOPA and 3-OMD from urine samples

Substance	Concentration ($\mu g\ ml^{-1}$)	Mean concentration recovered[a]
L-DOPA	2.0	1.87
C-DOPA	3.0	2.81

[a] $n = 5$.

the mobile phase flowed through the column and the flow cell and the nature of the cell construction made it very stable in the mobile phase with no leakage during its operating lifetime. The continuous washing process extended the life of the cell and minimised the start-up time. The working electrode was replaced every 7 days, but as such an electrode can be easily and cost effectively replaced, this problem of decreasing in sensitivity is not of major consequence.

Stability of urine samples kept in an ice bath was tested by making 5 consecutive injections of same urine sample over a period of approximately 3 h. There were no significant changes in the peak current between the first and the last samples. In addition, urine samples were stable for over a week when stored at -4°C. The recovery of the drugs from spiked urine samples was satisfactory (Table 3), and the concentration of the 'spiked samples' did not appear to influence the recovery of the residues in urine specimens.

4. Conclusions

The results demonstrate that L-DOPA, C-DOPA and their metabolites can be effectively resolved and quantitated in biological samples with a single on-line extraction and enrichment step followed by amperometric detection by employing a potential of $+0.8$ V at the working electrode. The micro-flow cell described here is simple to prepare and easy to manage. The cell has the advantage of small iR drop, virtually insignificant capacitive charging currents and a rapid mass transport to and from the working electrode. The method eliminates interferences from other endogenous components present in urine or plasma, it is not so subject to sample loss during transfer stages as off-line methods, and can be useful in routine biomedical and clinical investigations of these drugs and their metabolites. A work involving the use of this method in studying the different pharmacokinetic parameters and estimation of drug bioavailability in patients receiving different formulations containing a combination of these drugs and identification of impurities in these formulations will be conducted in our laboratory in the near future.

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