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DETERMINATION OF IMPURITIES IN LEVODOPA AND CARBIDOPA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

LINDA A. RIHBANY*
Winchester Engineering and Analytical Center, U.S. Food and Drug Administration, Winchester, MA 01890 (U.S.A.)
and
MICHAEL F. DELANEY
Department of Chemistry, Boston University, Boston, MA 02155 (U.S.A.)
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SUMMARY

The detection of 3-(3,4,6-trihydroxyphenyl)alanine and 3-methoxytyrosine in levodopa and methyldopa and 3-O-methylcarbidopa in carbidopa by high-performance liquid chromatography (HPLC) with electrochemical detection is described. An octyl-bonded reversed-phase column is employed with a buffered aqueous methanol mobile phase containing an "ion-pairing" reagent. All components are well resolved and sensitively detected by amperometric oxidation at a glassy carbon electrode maintained at +0.90 V *versus* Ag/AgCl. The impurities can be qualitatively characterized for identification purposes by hydrodynamic voltammetry, in which the peak height is observed for a range of oxidation voltages. The analysis of levodopa–carbidopa combination tablets is discussed.

INTRODUCTION

Levodopa (*l*-dopa, 3-hydroxy-L-tyrosine) and carbidopa (*S*-α-hydrazino-3,4-dihydroxy-α-methylbenzenepropanoic acid) are used in combination to treat Parkinson's disease[1]. The current *United States Pharmacopeia* (USP XX[2]) specifies maximum acceptable limits of two impurities in each of the two separate bulk powders. For levodopa, the USP XX requires that the major component be assayed by non-aqueous titration. The hydroxylated impurity 3-(3,4,6-trihydroxyphenyl)alanine and the O-methylated impurity 3-methoxytyrosine are measured by thin-layer chromatography (TLC). For carbidopa, the assay is also by non-aqueous titration. The non-hydrazine impurity methyldopa is measured by TLC and the O-methylated impurity, 3-O-methylcarbidopa, is determined by high-performance liquid chromatography (HPLC) on a 1-m pellicular cation-exchange column.

Each of these compounds contains a phenol or catechol functionality and thus should be amenable to sensitive and selective detection by amperometric oxidation[3].

We chose to develop an HPLC method with electrochemical detection (ElCD) which would be applicable to the determination of components in the bulk powders and in the combination dosage form. In combination with simultaneous ultraviolet absorption detection, both high and low levels of many compounds can be monitored.

Chromatographic separation and electrochemical detection of catecholamines in biological samples is a well developed technique with picogram detection limits for suitable compounds[4]. Few detection methods can compare for simplicity and performance[3]. With current improvements in chromatographic resolution[5] and detection sensitivity, standard methods of pharmaceutical analysis need to be re-examined and updated to insure acceptable drug quality and to maximize analytical laboratory efficiency.

In this paper we present a single HPLC–ElCD method which can detect and quantitate two drug substances and four specified USP impurities and which also indicates the presence of additional impurities. This approach is seen to be much more sensitive, simple and efficient than existing methodology.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a pump (Altex 110A single piston with pulse dampener, or Waters Assoc. 6000 dual piston), a valve injector with a 10-μl sample loop (Rheodyne 7125), a variable-wavelength ultraviolet absorption detector operated at 283 nm (DuPont Model 837) and an amperometric detector (Bioanalytical Systems TL-8A cell with an LC-3 controller). The electrochemical detector consists of a thin-layer flow cell with 3 mm diameter glassy carbon working and auxiliary electrodes and an Ag/AgCl reference electrode (4 *M* potassium chloride). For this study the working electrode was maintained in the oxidation mode at +0.90 V *versus* Ag/AgCl. Samples under 25 mg were weighed on an automatic electrobalance (Cahn Model 27). Chromatograms were recorded on a Linear Instruments dual-pen strip-chart recorder.

Materials

The water was deionized, distilled, suitable for HPLC. The methanol was HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), the sodium heptanesulfonate (Eastman Chemical, Rochester, NY, U.S.A.) was used as received and all other chemicals were ACS Reagent Grade. The drug substances and reference compounds were either from a commercial source or were purchased from USP. All sample solutions were prepared by dissolving the weighed solute in mobile phase with extra 1 *M* orthophosphoric acid added if needed to effect dissolution.

Chromatographic conditions

The HPLC column was a 12.5 cm × 4.0 mm I.D. 5-μm octyl-bonded reversed-phase column (LiChrosorb RP-8 Hibar) or a 25.0 cm × 4.0 mm I.D. 5-μm reversed-phase column (Ultrasphere). Both columns were commercially prepared. The mobile phase consisted of 16.5 g of sodium dihydrogen orthophosphate, 980 ml of water, 1.0 ml of 0.10 *M* disodium ethylenediaminetetraacetic acid, 20 ml of methanol and 1.2 ml of 0.5 m*M* sodium heptanesulfonate with the pH adjusted to 3.4 with 1 *M* orthophosphoric acid. The mobile phase was filtered through a 0.45-μm cellulose mem-

brane filter, vacuum degassed and used at ambient temperature with a flow-rate generally of 0.8 ml/min.

RESULTS AND DISCUSSION

Limits test of USP impurities

A reference chromatogram of all six known components is shown in Fig. 1 with corresponding retention information in Table I. The retention order for this reversed-phase separation can be explained on the basis of polarity. The primary amine levodopa elutes well before the less polar hydrazine-containing carbidopa. The more polar trihydroxyphenylalanine elutes before the dihydroxy levodopa and the extra methyl group in methyldopa causes it to be more retained relative to levodopa. The O-methylated levodopa derivative is more retained than levodopa and also elutes later than the dihydroxy methyldopa. Finally, the O-methylated carbidopa is most highly retained.

The mobile phase used is the same as that specified for the HPLC–ElCD analysis of epinephrine in combination with lidocaine which is described in USP XX Supplement 2[2], and is similar to another levodopa–carbidopa separation[6] as well as to that proposed for the separation of other catecholamines[7]. The EDTA is used to sequester any oxidizable metal ions. The effect on the separation of the "ion-pair" reagent concentration, pH and methanol for related compounds has been discussed elsewhere[7].

A dual detector chromatogram at 0.8 ml/min of a 10-μl injection of a 2.0 mg/ml sample of levodopa USP standard material is shown in Fig. 2. This would correspond to the analysis of a 100 mg levodopa–10 mg carbidopa tablet dissolved in 50 ml of solution. The UV detector is at its highest sensitivity (0.01 a.u.f.s.) while the electrochemical detector is at its lowest sensitivity (100 nA f.s.). Both impurities can be easily seen, but ElCD is much more sensitive.

TABLE I

CHROMATOGRAPHIC RETENTION TIMES

Compound	*Retention time (min)*	
	*Column 1**	*Column 2***
3-(3,4,6-Trihydroxyphenyl)alanine	5.5	2.9
Levodopa	6.9	3.5
Unknown impurity 1	9.1***	
Methyldopa	16.5	8.5
3-Methoxytrosine	19.3	9.8
Carbidopa	21.7	11.0
Unknown impurity 2	25.4***	
Unknown impurity 3	35.5***	
Unknown impurity 4	37.5***	
3-O-Methylcarbidopa	52.5	39.5

* 25-cm Ultrasphere column at 0.4 ml/min, increased to 1.0 ml/min after 30 min.

** 12.5-cm RP-8 column at 0.8 ml/min.

*** These impurities did not appear in fresh solutions.

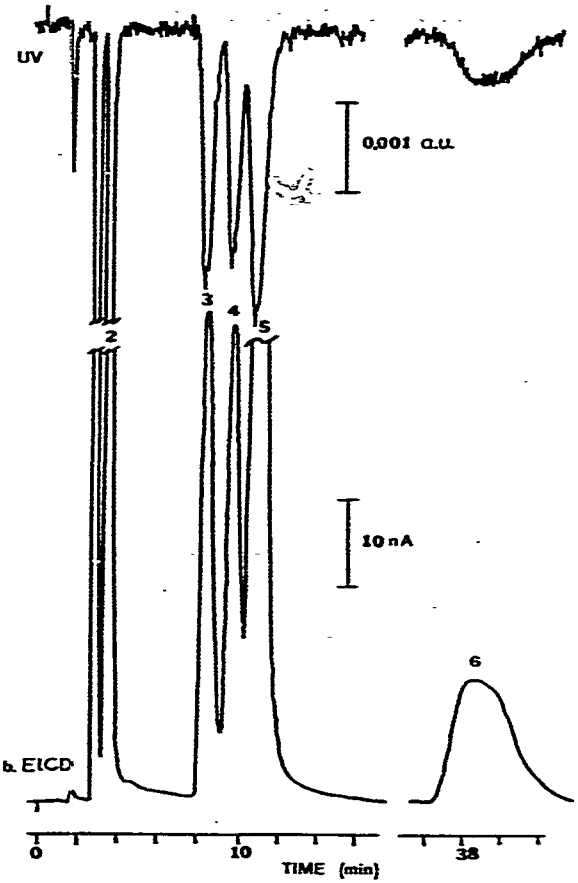

Fig. 1. Dual detector chromatogram of the six known components. (a) UV detection at 0.01 a.u.f.s.; (b) electrochemical detection of 100 nA f.s. Peaks: (1) trihydroxyphenylalanine; (2) levopoda; (3) methyldopa; (4) methoxytyrosine; (5) carbidopa; (6) methylcarbidopa.

Fig. 3 shows a chromatogram of USP standard samples of the two specified impurities at concentrations that are at the limit of USP acceptability for levodopa bulk powder. This corresponds to 2.0 μg/ml for trihydroxyphenylalanine and 10.0 μg/ml for methoxytyrosine. The EICD limit, estimated judiciously as 5% of the 5 nA f.s. sensitivity, is seen to be about 20 ng/ml for trihydroxyphenylalanine and 40 ng/ml for methoxytyrosine. This is a factor of 100–250 lower than the current USP limits, demonstrating the much higher sensitivities obtained with HPLC–EICD compared with conventional TLC.

A dual detector chromatogram at 0.8 ml/min of a 10-μl injection of a 0.20 mg/ml solution of USP primary standard carbidopa is shown in Fig. 4. This would correspond to the analysis of a 100 mg levodopa–10 mg carbidopa tablet dissolved in 50 ml of solution. The retention times of the two USP specified impurities are indicated. Fig. 5 shows a chromatogram of USP standard samples of the two impurities at the concentrations that are at the limit of USP acceptability for carbidopa bulk powder. This corresponds to about 1 μg/ml for both methyldopa and 3-O-methylcarbidopa.

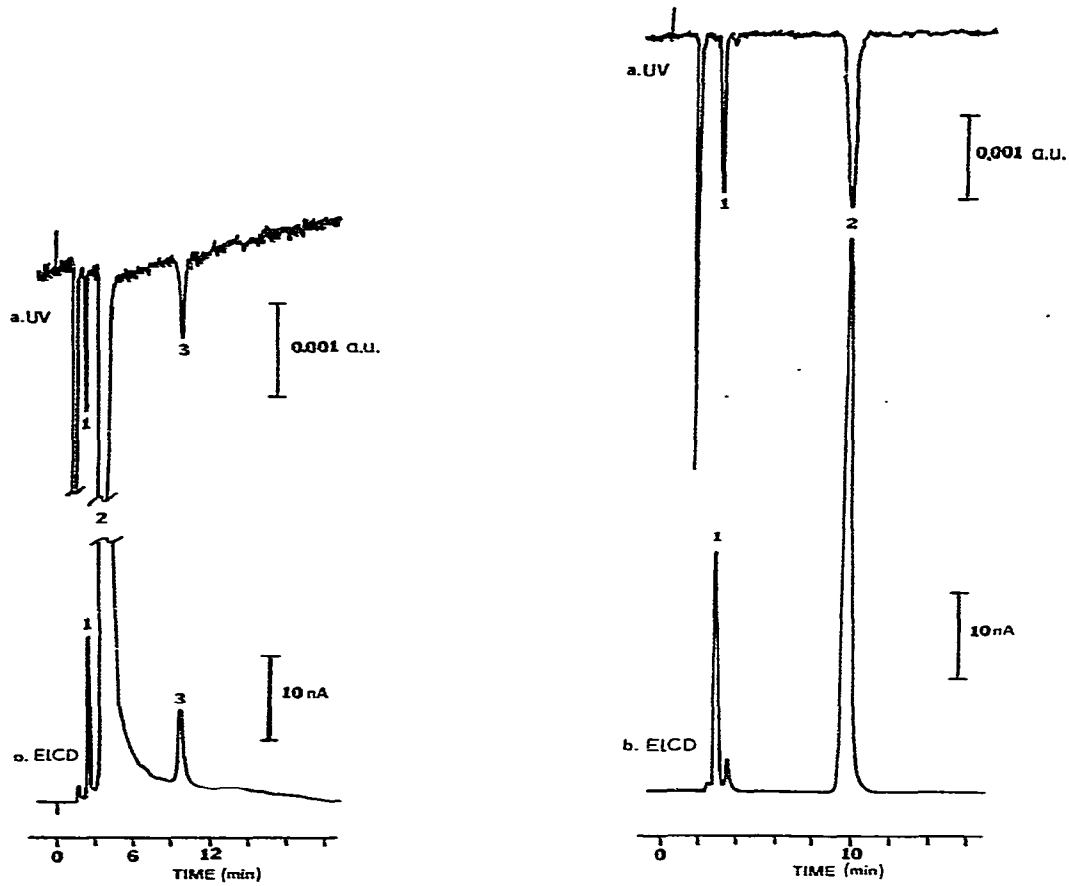

Fig. 2. Dual detector chromatogram of 2.0 mg/ml levodopa USP Standard. Peaks: (1) trihydroxyphenylalanine; (2) levodopa; (3) methoxytyrosine.

Fig. 3. Dual detector chromatogram of (1) 2.0 μg/ml of trihydroxyphenylalanine and (2) 10.0 μg/ml of methoxytyrosine.

Detection limits of about 80 ng for methyldopa and 60 ng for 3-O-methylcarbidopa can be obtained as 5% of the 5nA f.s. sensitivity. This is a factor of 10–20 lower than the current USP limits and could be further lowered by using a sample solution as concentrated as that used here for levodopa.

For comparison, Fig. 6 shows a dual detector chromatogram from a 0.20 mg/ml solution of a commercial source of carbidopa. In neither the USP reference sample nor the commercial material was the last impurity, 3-O-methylcarbidopa, observed.

Reproducibility

The reproducibility of each detector based on peak-height precision was evaluated by replicate injection of 100-ng amounts of 3-methoxytyrosine. For five replicates the UV absorption detector exhibited a coefficient of variation (relative standard deviation) of 0.96%. For the same injections the electrochemical detector

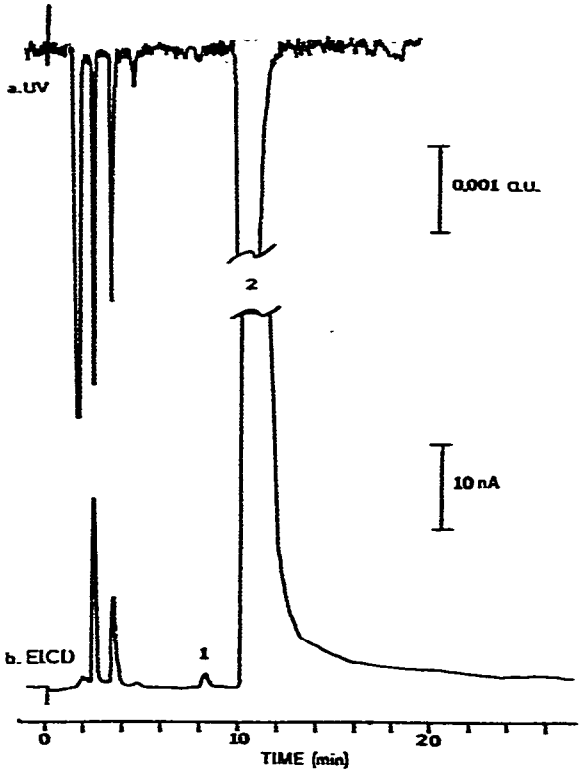

Fig. 4. Dual detector chromatogram of 0.20 mg/ml of carbidopa USP Standard. Peaks: (1) methyldopa; (2) carbidopa.

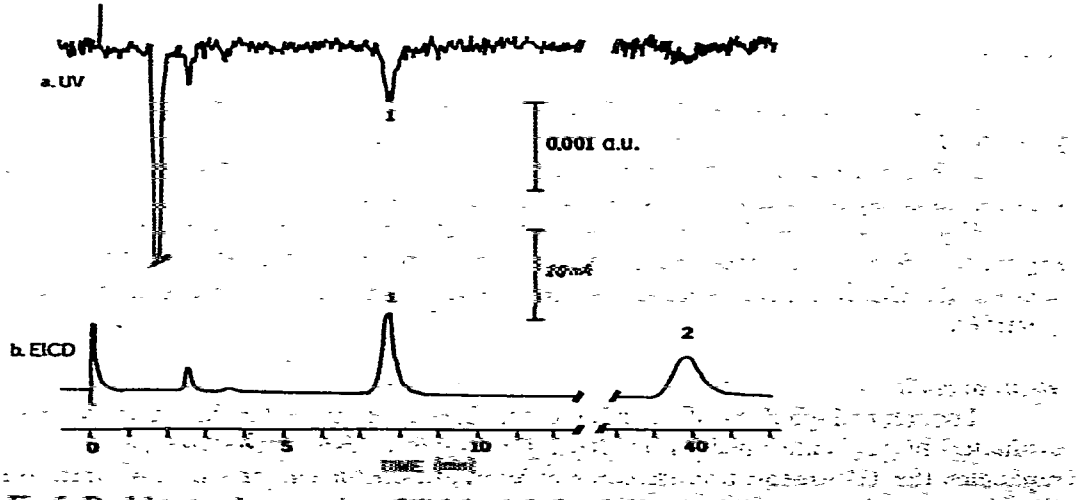

Fig. 5. Dual detector chromatogram of (1) 1.4 μg/ml of methyldopa and (2) 1.4 μg/ml of methylcarbidopa.

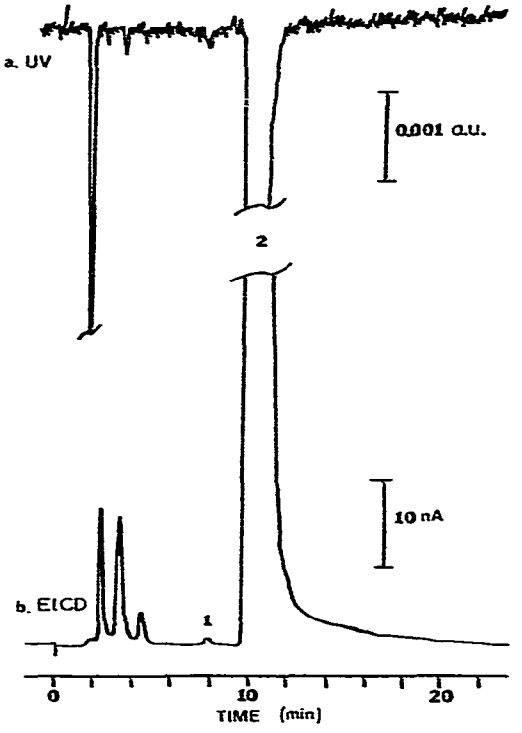

Fig. 6. Dual detector chromatogram of a 0.20 mg/ml solution of commercial carbidopa. Peaks: (1) methyldopa; (2) methylcarbidopa.

yielded a coefficient of variation of 0.28 %. This difference is mainly due to the fact the EICD trace was virtually noise-free whereas the UV peak had a signal-to-noise ratio of only about ten.

Qualitative identification

There is no guarantee in chromatography that two peaks from different samples that elute at the same time arise from the same substance. As the chromatographic efficiency is increased, such "coincidental coelution" becomes increasingly unlikely. However, it is advisable, when possible, to use some means of qualitative identification to insure that measured concentrations of impurity bear a high degree of validity. This identification generally becomes increasingly difficult as the level of the impurity decreases.

EICD for HPLC can provide such qualitative identification capability by observing the change in peak height *versus* applied oxidation potential. This is termed "hydrodynamic voltammetry"[3].

In this study a standard solution of each of the six compendial substances was repetitively injected while the detector operating voltage was increased from roughly 0.0 to 1.0 V in 25-mV increments. The resulting data are presented in Fig. 7 in which the peaks heights have been normalized with respect to the largest response observed for each compound. From these pseudo-polarograms characteristic half-wave potentials ($E_{1/2}$) have been extracted and are reported in Table II. The correlation between

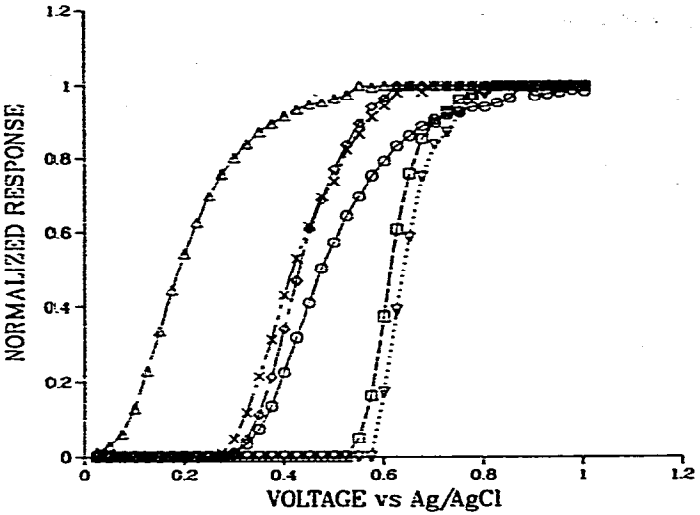

Fig. 7. Hydrodynamic voltammograms of the six compounds. ○, Levodopa; △, 2,4,6-trihydroxyphenylalanine; ×, carbidopa; ◇, methyldopa; ▽, 3-O-methylcarbidopa; □, 3-methoxytyrosine.

chemical structure and use of oxidation is clearly seen: The trihydroxy compound has the lowest $E_{1/2}$, the three dihydroxy species have intermediate $E_{1/2}$ and the two monohydroxy substances have the largest $E_{1/2}$. This information can be used to provide qualitative information in support of the assignment of identity of impurity peaks.

Degradation study

It was our observation that additional impurity peaks developed in the HPLC–EICD trace for standard solutions which had been allowed to stand for more than a day (Table I). Although we have not pursued this to the point of identifying these impurities, we have monitored the behavior of these components for a week using a solution containing all six target compounds at the 1–2 mg/ml level.

Impurities 1, 2 and 3 appear in solutions that are a day old. The response due to impurity 1 became > 100 nA after 6 days and remained high, while impurity peak 2 reached a maximum of 16 nA on day 3 and fell off thereafter. Impurity 3 reached a level of 10 nA on day 8. Impurity 4 appeared on day 3 and reached 1.5 nA on day 8.

TABLE II

HYDRODYNAMIC VOLTAMMETRIC HALF-WAVE POTENTIALS

Compound	$E_{1/2}$ *(V vs Ag/AgCl)*
3-(3,4,6-Trihydroxyphenyl)alanine	0.19
Carbidopa	0.42
Methyldopa	0.43
Levodopa	0.48
3-Methoxytrosine	0.61
3-O-Methylcarbidopa	0.64

The only significantly noticeable change in peak height for the six compounds was for 3,4,6-trihydroxyphenylalanine, which initially had a peak height >100 nA but which had fallen to 12 nA after 8 days. A solution containing only 3,4,6-trihydroxyphenylalanine was prepared. This solution developed impurity peaks upon standing which appear to correspond to impurity peaks 2 and 3 in Table I. This initial degradation study demonstrates the potential of HPLC–ElCD to monitor the storage and breakdown pathways of oxidizable organic compounds.

CONCLUSIONS

The combination of the high sensitivity of amperometric detection with the superb resolving power of liquid chromatography has been seen to be used advantageously to provide a simple and effective means by which to determine the amount of impurities in commercial samples of levodopa and carbidopa as specified by the USP[2]. The method can supersede four existing methods for the specified impurities and is sensitive enough for single tablet analysis.

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