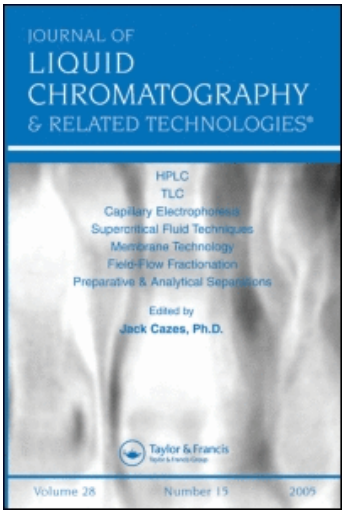


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# DETERMINATION OF THE INFLUENCE OF BORATE ION ON THE DEGRADATION OF L-α-METHYLDOPA USING RP-HPLC WITH PHOTODIODE ARRAY DETECTION

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# DETERMINATION OF THE INFLUENCE OF BORATE ION ON THE DEGRADATION OF L-α-METHYLDOPA USING RP-HPLC WITH PHOTODIODE ARRAY DETECTION

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## ABSTRACT

The degradation of the drug L-α-methyldopa (MD) in alkaline solutions was studied by Reversed-Phase High Performance Liquid Chromatography. It was found that the presence of borate ion in the solution inhibited the formation of MD-melanin, the final product of degradation. It complexes with both MD and intermediates of the pathway, maintaining them longer in the medium. Through the use of a synthetic standard of a half-way intermediate of the oxidation pathway, 5,6-dihydroxy-2-methylindole (Me-DHI), and examination of the UV/vis spectra of the chromatographic peaks, it was possible to demonstrate the stabilization of a later stage degradation intermediate in the presence of borate ion.

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The inhibition of melanin formation may be correlated to the stabilization of this intermediate. Hence, this mechanism could be generalized and applied to biogenic catecholamines, such as dopamine and L-dopa, the natural precursor of mammalian melanin.

## INTRODUCTION

More than 30 years after the introduction of L-3-(3,4-dihydroxyphenyl)-2-methylalanine (L-α-methyldopa) (MD) in therapeutics by Merck Sharp & Dohme (Aldomet®), this drug still is one of the most useful hypotensive agents. Of recognized efficacy, it has been clinically employed in all forms of arterial hypertension. Owing to its phenolic nature, MD undergoes oxidation in alkaline media to a polymeric melanin-like pigment, in an analogous way to L-dopa, the natural precursor of brain catecholamines and mammalian melanin.(1) MD can also serve as a substrate for pigment formation in the melanocytes of hair follicles;(2) and its incubation in human blood plasma produced plasma soluble rheomelanins after 24 h at 38 °C.(3) This tendency for degradation frequently generates difficulties with the stability of MD dosage forms.(4-7)

Borate ion is a known chelating agent for 1,2-diols, including catechols.(8,9) A study performed in the middle of the fifties showed that borate exercised a high degree of inhibition on the oxidation of L-dopa and other catecholamines.(10) Another investigation, realized in the early seventies, produced evidence that the borate ion inhibited the MD oxidative process.(11) The authors also verified that it could interrupt the process at a middle or higher point by chelating with the degradation intermediates. Moreover, at higher pH values polymerization of the quinonoid intermediate produces a lower molecular weight water-soluble polymer, which is resistant to further oxidation and polymerization.(11)

In this paper, we focused on the effect of borate ion in slowing down MD oxidation. Our aim was to carry out a systematic study using High Performance Liquid Chromatography with photodiode array detection (PDA) as the analytical method, which permits more precise identification of the mode of action of borate ion on the inhibition of MD degradation. The UV/vis spectra of the eluted compounds (*via* PDA) provided valuable information about their nature. In addition, we utilized a previously synthesized standard of 5,6-dihydroxy-2-methylindole (Me-DHI), the last detectable monomeric intermediate of MD degradation, which aided us by comparing its degradation profile to that of MD. The chromatographic system employed was based on previous studies by Metwally and Belal, which described a stability-indicating method for MD but did not fully clarify the mechanistic aspects of the degradation pathway.(12,13)

## EXPERIMENTAL

### General

Infrared spectra were obtained on a Nicolet 205 FT-IR spectrometer; $^{1}H$ (200.1 MHz) and $^{13}C$ (50.3 MHz) NMR spectra were determined on a Bruker AC-200 F spectrometer using TMS as internal standard; UV spectra were recorded on a Shimadzu1601 spectrophotometer; melting points (uncorrected) were determined on a Kofler apparatus (Reichert) and mass spectra were recorded on an HP GC/MS coupling system, consisting of an HP 5890 chromatograph, with a 50 m HP-5 (SE-54®) column and $H_2$ as carrier gas and an HP 5970 mass spectrometer, with electron impact at 70 eV.



### Reagents

Methyldopa (MD) USP reference standard was purchased from Merck & Co., methanol (HPLC grade) and acetic acid from E. Merck, and sodium heptanesulfonate (98 %) from Aldrich. Deionized water was obtained by using a Millipore Milli-Q purification system. Sodium hydroxide, boric, acetic, and phosphoric (88 %) acids, and other reagents were all of analytical grade and were used as received.

### Apparatus

HPLC analyses were carried out on a Hewlett-Packard instrument model 1090, equipped with a photodiode array detector set at 280 and 300 nm, an HP 3396A integrator and an HP Ink Jet printer, from which were obtained the UV/vis spectra of chromatographic peaks. Separations were achieved on a 250 × 4.6 mm Shim-Pak $C_8$ (5 μm) analytical column (Shimadzu) at 36°C. Injections were made through a Rheodyne 7125 injection valve with a 20-μL sample loop.

### Mobile Phase

The mobile phase(12,13) consisted of methanol: water (18:82) containing 2% (v/v) acetic acid and 5 mM sodium heptanesulfonate operating at a flow-rate of 1.6 mL/min. The pH was 2.60 ± 0.05.

Sample solutions were filtered before injections through a 0.45 μm Millipore filter (Millex) and the mobile phase was vacuum filtered by passing it through a 0.50 μm Millipore Teflon® membrane and degassed by ultrasonic bath before use.

The pH measurements of both mobile phase and reaction solutions were made in a Procyon pH-meter using a glass electrode and Ag/AgCl as reference, previously calibrated with Merck buffers (pH 4.00 and 7.00).

## Calibration Graph and Quantification

An accurately weighed 90-mg sample of MD was dissolved in 200 mL of 0.01N $H_2SO_4$ in a 500-mL volumetric flask and then brought to volume. Serial dilutions were made to the following MD concentrations (μg/mL): 180, 144, 90, 72, 45, 36, 18, and 9. Each standard solution was injected in duplicate and the peak areas were recorded. The slope and the intercept of the calibration graph were obtained by linear regression of peak areas versus concentration, with a regression coefficient of over 0.999. The detection limit of 0.4 μg/mL was established. MD concentrations in the samples were calculated by comparing the peak area with the standard concentrations, through the plotted calibration graph.



## Degradation of MD and Me-DHI

The degradation reactions were effected by allowing the substrate to remain in contact with an oxygen-saturated solution at an appropriate pH value (autoxidation).

10 mg of the component were analytically weighed and dissolved in the solution to be degraded in volumetric flasks of appropriate volume, according to the desired final concentration. When necessary, the pH was rapidly adjusted to the required value with diluted sodium hydroxide. After an appropriate time period, 10 mL of the sample were transferred into a 25-mL volumetric flask and quenched with 5-10 mL of 0.1 N $H_2SO_4$. The mixture was then adjusted to volume with deionized water, resulting in a solution with pH of about 2.

The MD and Me-DHI peaks were identified by co-elution with the standards and by the overlaying of the UV/vis spectra.

The synthesis of 5,6-dihydroxy-2-methylindole (Me-DHI) was based on the procedure described by Young et al.,(1) with minor modifications. These included the addition of excess sodium dithionite and 6N HCl to pH ~ 5 before the extraction step, to maintain Me-DHI more stable in solution. After recrystallization from ether/benzene/hexane, the purified product was obtained as colorless crystals in 10% yield. IR (KBr): 3444 $cm^{-1}$ (ν OH); 3390 $cm^{-1}$ (ν NH). $^1$H NMR (DMSO-$d_6$ + $CDCl_3$): δ (ppm) = 2,33 (s, 3H, $CH_3$); 5,92 (s, 1H, =CH); 6,81 and 6,90 (2s, 2H, H-4 and H-7); 7,43 and 7,44 (2s, 2H, 2OH - exchanged with $D_2O$); 8,88 (s, 1H, NH - exchanged with $D_2O$). $^{13}$C NMR (DMSO-$d_6$ + $CDCl_3$): δ (ppm): 13,59 ($CH_3$); 96,94 (C–C=C); 98,80 (H–C=C); 104,23 (H–C=C); 121,89

(H–C=C); 130,63 (N–C=C); 133,57 (N–C=C); 139,55 (C– OH); 141,10 (C–OH). MS- m/e: 164 ($MH^+$, rel. intensity 100%). UV (EtOH): $\lambda_{max}$ = 276 nm (log ε = 3,73); 306 nm (log ε = 3,92). M.P. 178 $^0$C (dec).

## RESULTS AND DISCUSSION

The following results show that care is necessary in choosing the buffer solution to be used for the degradation of MD.

Figure 1 shows the chromatographic profile of MD degradation carried out in aqueous sodium hydroxide or in a buffer composed of 0.04 M phosphate, acetate and borate (Britton-Robinson buffer),(14) at two pH values for each system: 9.15 (1a and 1b) and 11.34 (1c and 1d). In 1a and 1c the reaction system was the Britton-Robinson (B.R.) buffer, while in 1b and 1d it was aqueous sodium hydroxide. There is a clear difference in the products' distribution, as well as, in the consumption of MD, as indicated in Table 1. The color development observed in the solutions was coherent with the HPLC results. For the same pH value and degradation time, the reaction darkening was always stronger in NaOH solutions than in the B.R. buffer. At pH 9.15, for example, the NaOH solutions became almost black after 1 week and revealing insoluble melanin formation, whereas the buffer solutions were light brown with no precipitate. Furthermore, in the buffer, the time elapsed until appearance of the first perceivable color was longer than in the NaOH solutions.

Assays performed with Me-DHI showed a similar visual effect. Figure 2 shows this comparison, at a pH of about 10.5. Note that the substrate is extremely unstable at this pH, even in the buffer. The last peak, eluted at about 8.8 min, is notable in that it persists in the buffer (2a, c, and e) but is essentially absent in aqueous NaOH (2b and d), even in the first reaction minutes. The UV/vis spectrum of this peak compares with that seen in MD degradation, having also a similar retention time (see Fig. 2c inset *vs.* 1a and 1e), clearly corresponding to the same compound. Therefore, the intermediate of MD degradation, which elutes shortly before unchanged MD, comprises a later stage of the pathway, since it is a derivative of Me-DHI degradation. Nevertheless, even in the B.R. buffer, its peak was not detected at above pH 11 (Fig. 1c).

With regard to its identity, one can suppose the presence of orto-hydroxyl groups in the ring, from the fact of being stabilized by $H_3BO_3$. However, the enone (or quinone) function can also be present at some site of the molecule, as is suggested by the relatively weak, broad band centered at 330-40 nm.(15) There is also an absorption band with max. at ~290 nm, in agreement with data of acetylated dimers and trimers of Me-DHI.(16) The structure of one of them is shown in Figure 3, as an example.

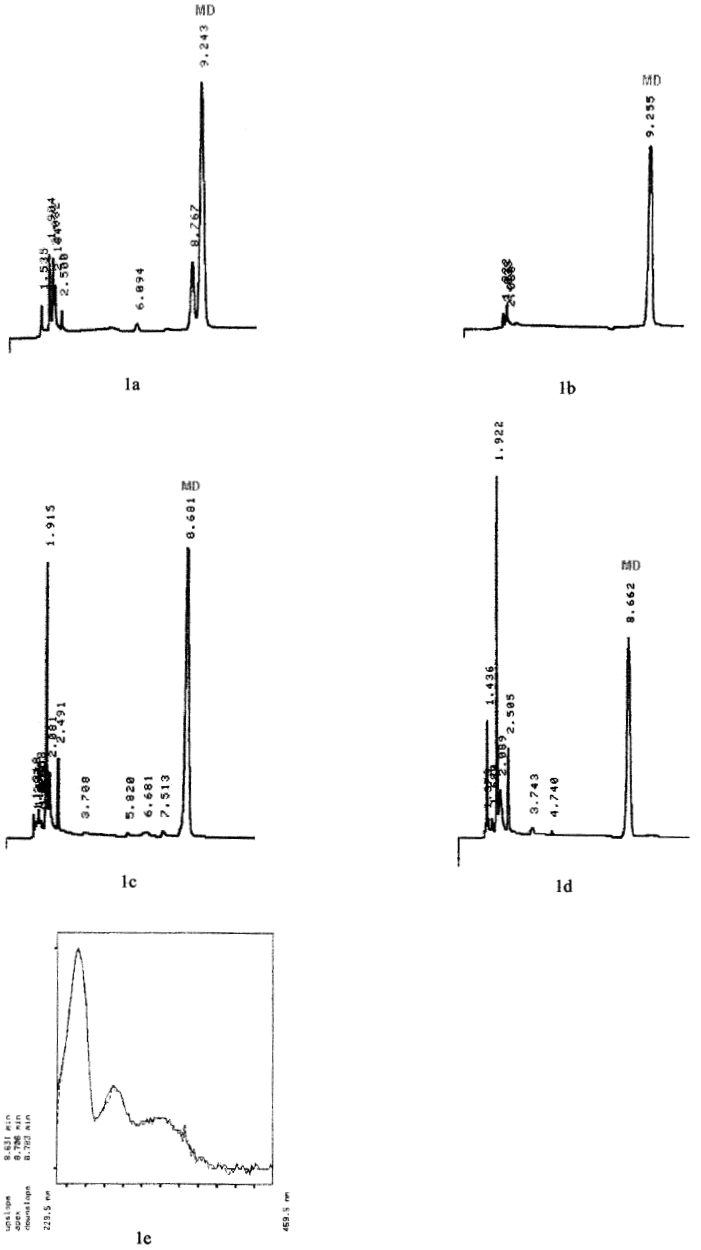


***Figure 1***. Comparison of MD degradation in aqueous NaOH solution versus in B.R. buffer. (a) Degradation in B.R. buffer at pH = 9.15, after 30 h; (b) As in (a), but in aq. NaOH; (c) Degradation in B.R. buffer at pH = 11.34, after 3.8 h; (d) As in (c), but in aq. NaOH (e) UV/vis spectrum of the peak before MD in (a) ($R_t$ 8.77 min): $\lambda_M$ = 252, 289, and 340 nm. In (a) and (b), [MD] = 120 ppm; in (c) and (d), [MD] = 150 ppm.

***Table 1.*** Effect of Buffer on MD Degradation (Methyldopa in Solution (%))

| | pH 11.34* | | | pH 9.15** | |
|---|---|---|---|---|---|
| Elapsed Time (h) | B.R. bf. | NaOH | Elapsed Time (h) | B.R. bf. | NaOH |
| 1.2 | 80.4 | 65.5 | 5.5 | 95.0 | 83.3 |
| 3.8 | 59.7 | 47.6 | 30 | 62.5 | 45.6 |

* B.R. buffer : pH = 11.35; aq. NaOH : pH = 11.32.
** B.R. buffer : pH = 9.14; aq. NaOH : pH = 9.16.



As commented before, the borate ion is a well-established chelating agent for catechols, which can be related to the greater MD stability in the buffer system used. The method employed permits visualization of the influence of this ion on the stabilization of a major intermediate, whose peak is absent in the chromatograms corresponding to the aqueous hydroxide systems. This confirms the results reported in the seventies, where the blockage of the oxidation pathway by borate ion had been proposed.(11) On that occasion the autoxidation was accomplished in the pH range 5-10. In the present study, we also found that at pH above 11 the borate ion exerts a pronounced effect.

An important implication of these findings is that the presence of borate offers the prospect of isolating and characterizing, by preparative HPLC, for example, an intermediate which accumulates in the medium and can play a key role in the melanogenesis process, since melanin formation is greatly inhibited when this component is stabilized. Moreover, from the pharmacotechnical point of view, this study reinforces the idea of adding boric acid or derivatives to pharmaceutical formulations containing methyldopa (or congeners), in order to increase its stability.(7)

The choice of the Britton-Robinson buffer was based on its earlier use in a kinetic study of MD degradation, covering the pH range 2-12, in which the first and second order rate constants were obtained.(13) The authors' main objective was to ascertain the influence of pH on MD consumption (pH-rate profile). Since the Britton-Robinson buffer has such a remarkable effect in slowing down MD degradation, it may not be appropriate for evaluating the role of pH in catechol/catecholamine oxidation. The use of more inert buffers, such as bicarbonate and acetate would be recommended in this case.(10) Thus, the kinetic parameters previously obtained may be misleading. For example, the authors conclude that in the pH range 2.0-8.0 the degradation is pH-independent, the considerable variation in results being attributed to the difficulties inherent in measuring rate constants for very slow reactions.(13) However, the current study suggests care in this evaluation. In the presence of borate the reaction is so slow that it may give rise to this kind of interpretation. We effected MD degradation at pH 4.3 and pH

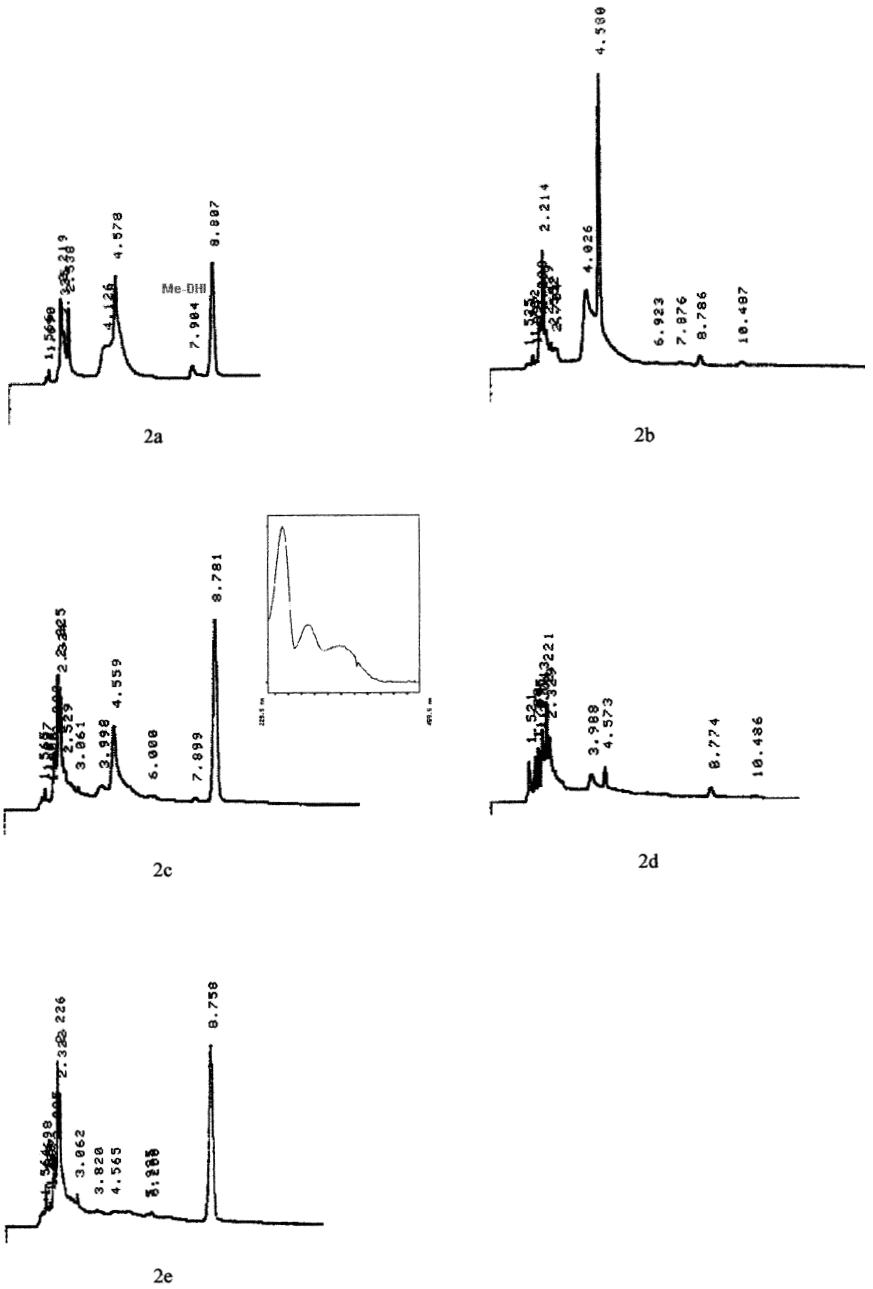


***Figure 2***. Chromatograms of 100 ppm Me-DHI at pH ~10.5. (a) After 5 min in B.R. buffer; (b) After 10 min in aq. NaOH; (c) After 45 min in B.R. buffer (inset: UV/vis spectrum of the last peak); (d) After 50 min in aq. NaOH and (e) After 80 min in B.R. buffer. $\lambda_{det}$ = 280 e 300 nm. The retention time of Me-DHI is 7.8-9 min.

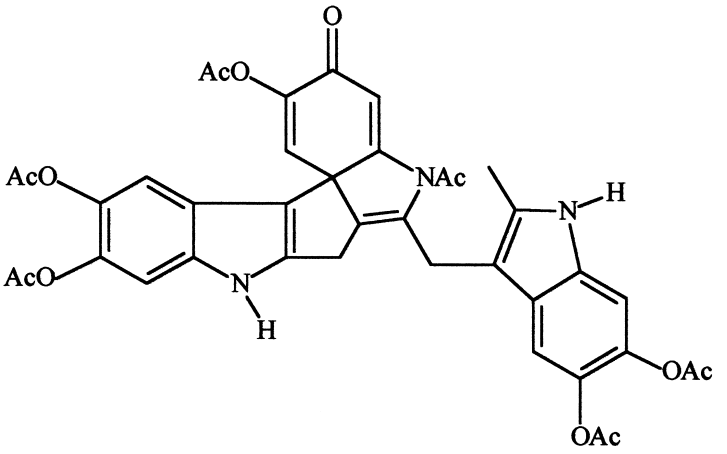


***Figure 3***. Example of an enzymatic degradation product of Me-DHI.(16)



6.8, in acetate buffer, and although no kinetic study was made, the difference is such that one can suggest that the process is pH-dependent even in the acidic range:

| | | MD remaining (%) |
|---|---|---|
| 0.1M acetate buffer pH 4.3 | → | 96.6 (after 68 hours) |
| 0.1M acetate buffer pH 6.8 | → | 89.7 (after 48 hours) |

A visual inspection of these solutions is coherent with the numbers. At pH 4.3 the darkening is slower and less intense than at pH 6.8.

In a study involving the dopachrome decay - the slow step of oxidation pathway - less stabilization was found when a borate buffer was used.(17) Additionally, it was shown that increasing borate concentration, resulted in a decrease of the life time of dopachrome. This result is not opposite to ours. It just shows that inhibition of melanization by borate ion should not be explained in terms of the early stages of the pathway (up till formation of dihydroxyindole).

Other studies focus on the chemical ($NaIO_4$) and enzymatic (tyrosinase) oxidation of L-dopa using high performance liquid chromatography with electrochemical detection (HPLC-ED),(18,19) a method which has the advantage of being very sensitive and selective, differentiating oxidized from reduced species. The stability of dopamine was also investigated by this technique.(20) The authors were able to identify early stage degradation intermediates, through indirect methods. However, if the objective is to investigate also the later stages of the process, as in the autoxidation in alkaline medium, the ED can fail, since the

formed species tend to be electrochemically inactive.(1) Thus, PDA detection may be more suitable for such analyses, and using HPLC-PDA rather than simple spectrophotometry(11,21) has the advantage of providing the UV/vis spectrum of each separated individual component, thus, making it easier to identify them and monitor their dynamic changes during reaction.

We believe these findings could be extended to other cases, such as enzyme-mediated reactions using L-dopa as substrate, in the further clarification of the mechanism of melanin biosynthesis.

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