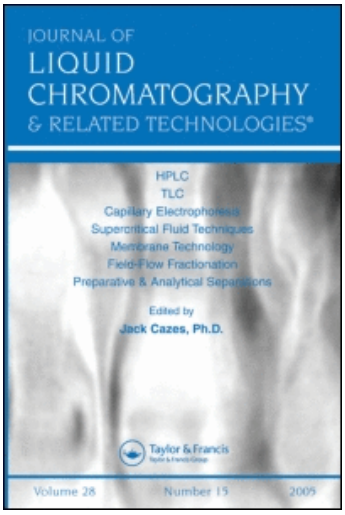

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Peter Rembischevski[a]; André L. Gemal[b]
[a] Instituto de Tecnologia em Fármacos, Rio de Janeiro, Brazil [b] Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, Brazil

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Peter Rembischevski,[1] André L. Gemal[2]

[1] Fundação Oswaldo Cruz
Instituto de Tecnologia em Fármacos
Sizenando Nabuco 100, Manguinhos
21041-250, Rio de Janeiro, Brazil

[2] Instituto de Química
Universidade Federal do Rio de Janeiro
Cidade Universitária
CT-Bloco A
21949-900, Rio de Janeiro, Brazil

ABSTRACT

The present work is devoted to the analysis of the phenomenon of "double peaks" of aminoacids in High Performance Liquid Chromatography. Therefore, we have carried out some experiments in order to confirm that the appearance of several peaks from the injection of a single compound was related to different ionic forms of this compound. It could be shown that elution of multiple peaks for L-α-methyldopa did not refer to its degradation products, but to the different ionic species present in the media. The same occurred with its derivative 3-O-methylmethyldopa and with dl-phenylalanine.

The separation between ionic forms was possible due to the use of sodium heptanesulphonate, an anionic ion-pairing reagent, which selectively interacts with different charged species. These findings show that equilibrium species can be separated by an appropriate analytical method, depending on the employed conditions.

INTRODUCTION

The elution of multiple peaks from the injection of a single component, in HPLC, is not a very usual phenomenon. The causes can be several, depending on the case, i.e., nature of the compound and solvents used. Frequently, ionizable compounds originate broad and tailing peaks, if the employed chromatographic elution system is not appropriate. We have found pertinent literature dealing with mechanistic studies involving separation of ionogenic substances (such as amines and aminoacids), mainly by ion-pair RP-HPLC.[1-9] Most of these works stated that equilibrium forms of ampholytes would not likely to be separated chromatographically. Instead, a single peak should elute, with a mean value of the capacity factor (k'), depending on the contribution of each specie present in the equilibrium in a certain pH value.

However, in the analysis of amines and aminoacids some authors have obtained good peaks' resolution from the injection of a pure substance.[3,10] Accordingly, Lundanes & Greibrokk suggested the hypothesis that dimeric forms of the aminoacids would be eluting with different retention time of the respective monomer.[3] They alleged that the size of the first peak compared with that of the second peak seemed to be disproportional to the pI values of the aminoacids. On the other hand, Honigberg et al. preferred to attribute the multiple peaks to the discrimination between different ionic forms.[10]

In the present paper, we propose to investigate the occurrence of several peaks from injection of a single Phenyl-derivative by means of methodically planned experiments. The starting point of our analysis was with the important hypotensive agent L-α-methyldopa (MD) [L-3-(3,4-dihydroxyphenyl)-2-methylalanine]. The occurrence of multiple peaks with MD, in HPLC, was already observed with a buffered mobile phase, pH > 8[10].

Since MD is a very susceptible molecule to chemical oxidation, specially in alkaline media,[11,12] the production of multiple peaks in the corresponding chromatograms could be confusingly associated to its degradation products. The authors of the above paper[10] attributed the multiple peaks as an "indicative of the several species of methyldopa possible in basic media."

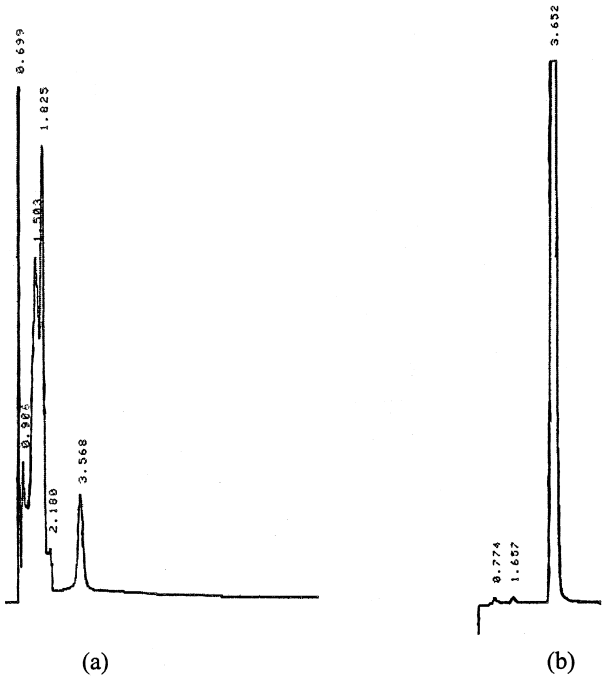


Figure 1. Chromatograms of 150 μg/mL MD dissolved in : (a) - methanol and (b) - the mobile phase. This was for both (a) and (b): H_2O: MeOH (82 : 18) with 2 % acetic acid and 5 mM of C_7-SO_3Na. Column: Hypersil ODS 100 × 4.6 mm; flow rate 1.5 mL / min; t_R in (a) = 3.568, 2.180, 1.825, 1.503, and 0.906 min; t_R in (b) = 3.652 min.

We obtained multiple peaks from methanolic solutions of MD and carefully demonstrated that these peaks were not related to its degradation products, thus elaborating arguments that justify the hypothesis of ionic forms separation.

Other aminoacids were analysed in the same conditions, also undergoing peak duplication. They were dl-phenylalanine and 3-O-methylmethyldopa (MMD), an intermediate of the MD industrial synthesis. We based our experiments on the use of sodium heptanesulphonate (anionic ion-pairing) which distinctly interacts with cations, anions and neutrals.

Another powerful tool was the use of photodiode array detector (PDA), which allows the obtaining of ultraviolet and visible (UV/Vis) spectra of the corresponding peaks from the same chromatographic run.

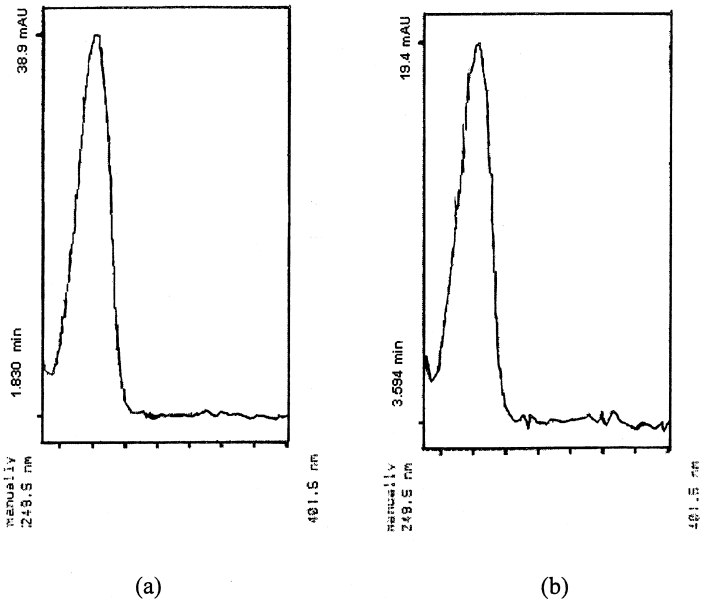


Figure 2. UV/Vis spectra of the eluted peaks in 1(a). λ_M = 280 nm in both (a) and (b).

EXPERIMENTAL

Reagents

DL-Phenylalanine (99 %) was purchased from Aldrich, methyldopa (MD) and 3-O-methylmethyldopa (MMD) from Merck & Co. (USP reference standard). Methanol (HPLC grade) and acetic acid were purchased from E. Merck and sodium heptanesulphonate (98 %) from Aldrich. Deionized water was obtained by using a Millipore Milli-Q system.

Liquid Chromatograph

The HPLC analyses were carried out on a Hewlett-Packard instrument model 1090, equipped with a photodiode array detector set at 267 nm to phenylalanine and at 280 nm to MD and MMD. The eluted peaks were integrated by an HP 3396A integrator and the corresponding UV/vis spectra were printed by an HP Think Jet printer.

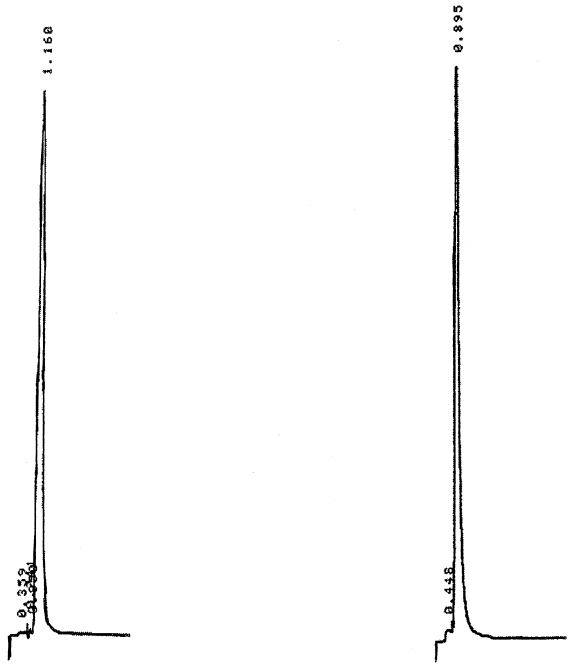


Figure 3. Chromatograms of MD dissolved in methanol, eluted in the mobile phase: (a) - H_2O: MeOH (82:18) and (b) - H_2O: MeOH (50:50). Column: Hypersil ODS 100 × 4.6 mm; flow rate 1.5 mL / min; t_R in (a) = 1.160 min; t_R in (b) = 0.895 min.

Separations were achieved on a 100 × 4.6 mm Hypersil (HP) C_{18} (4 μm) or 125 × 4 mm Lichrospher 100 (Merck) C_{18} analytical columns at 36°C. Injections were made through a Rheodyne 7125 injector fitted with a 20-μL sample loop.

Mobile Phase

Unless otherwise indicated, the mobile phase was constituted of methanol: water (18 : 82) containing 2 % (v/v) acetic acid and 5 mM sodium heptane-sulphonate operating at a flow-rate of 1.5 mL/min.[12,13]. The mobile phase pH was 2.60 ± 0.05. It was vacuum filtered by passing it through a 0.50 μm Millipore teflon® membrane and degassed in a ultrasonic bath before use.

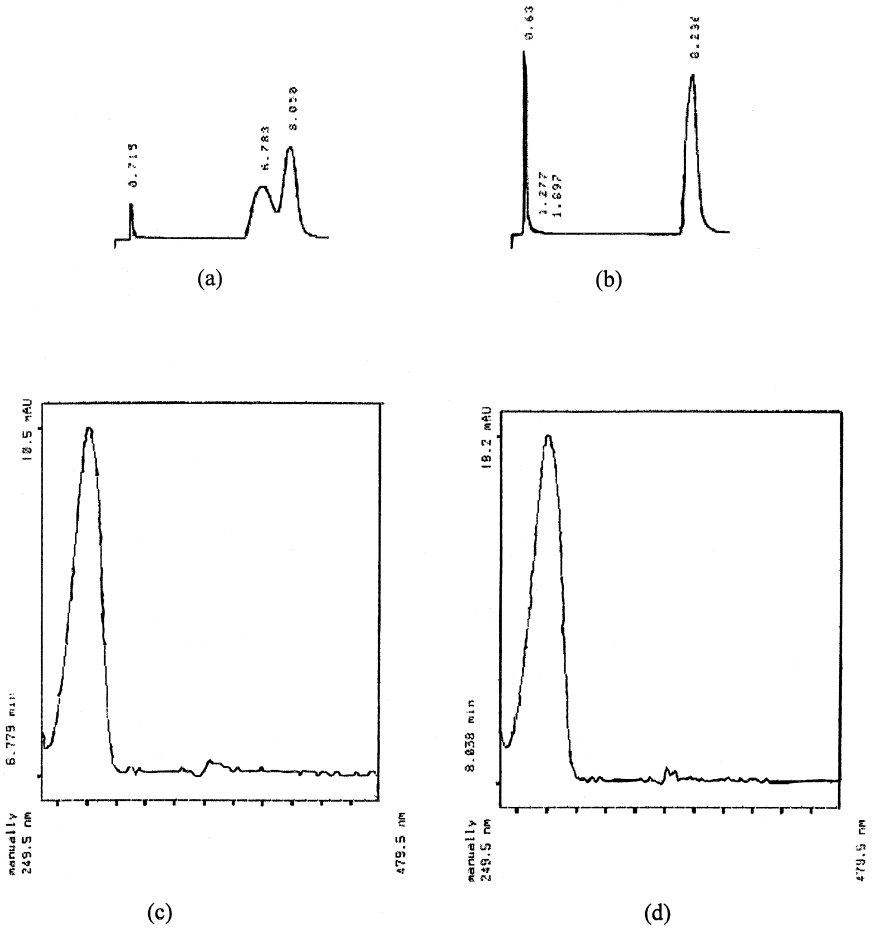


Figure 4. HPLC-PDA analysis of 3-O-MMD dissolved in: (a) - methanol and (b) - 0.05 M H_2SO_4. (c) and (d) - UV/Vis spectra of the two peaks in (a); λ_M = 280 nm in both (c) and (d). Mobile phase: H_2O: MeOH (82: 18) with 2 % acetic acid and 5 mM of C_7-SO_3Na. Column: Hypersil ODS 100 × 4.6 mm; flow rate 1.5 mL / min; t_R in (a) = 8.050 and 6.783 min; t_R in (b) = 8.236 min.

Preparation of Sample Solutions

The component was dissolved in MeOH, 0.05 M H_2SO_4 or mobile phase, at 100 μg/mL (unless otherwise indicated) by simple dissolution method and filtered through a 0.45 μm Millipore filter (Millex) before injection. The chromatographic pattern alters depending on injection solvent, as shown.

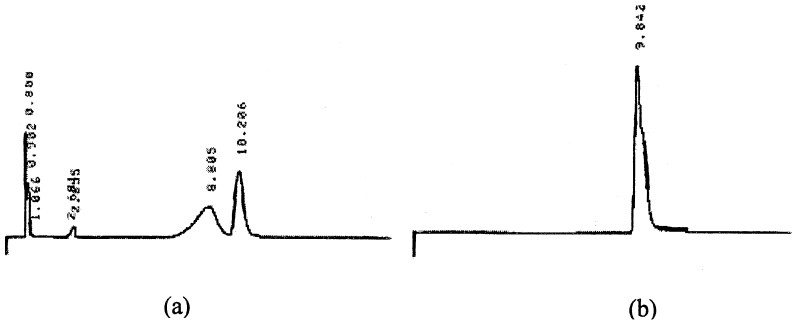


Figure 5. Chromatograms of phenylalanine dissolved in: (a) - methanol and (b) - mobile phase. Column: Lichrospher ODS 125 × 4 mm; mobile phase and flow rate as in figure 1; t_R in (a) = 10.206, 8.805, 1.066 and 0.962 min; t_R in (b) = 9.842 min.

RESULTS AND DISCUSSION

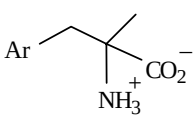
Two different freshly prepared solutions of MD were analysed, a) in MeOH and b) in the mobile phase. In the first case (Figure 1a) it could be noticed that upon detection at 280 nm at least four peaks were produced, while in the second case (Figure 1b) only one peak was produced, thus showing the importance of the solvent in the formation of detected artifacts. UV/vis spectra of the compounds eluted at 1.825 min and 3.568 min showed the same pattern, therefore confirming their identity (Figure 2). The same spectrum was also obtained for the peak eluted at 1.503 min (not shown). Another relevant supporting datum is that the sum of individual peak areas in 1a corresponded well with the area under the single peak in 1b. However, when the run was done with a mobile phase without the ion-pair reagent, only a single peak was eluted (Figure 3). This datum is consistent with the idea of trapping different ionic forms, since in the absence of the ion-pair reagent there was no discrimination. Therefore, this reagent should be selectively interacting with the charged species. It is worthy to point out that the retention of MD is greater in 3a than in 3b, in agreement with the relation between mobile phase polarities (a > b), considering the reversed-phase system.

Other compounds were tested in order to ascertain whether or not the case was specific to methyldopa. Figure 4a shows the elution pattern of 3-O-methylmethyldopa (MMD) dissolved in MeOH, which was different when this compound was dissolved in aqueous H_2SO_4 (4b). Analogously to MD, the two peaks eluted in 4a have showed identical absorption spectra (4c and 4d). In this case, there is no possibility of degradation, since MMD is stable even in $AgNO_3$-NH_3 or 0.1 N NaOH.

The same phenomenon was also observed with dl-phenylalanine. (Figure 5). Separation took place when the sample was dissolved in methanol (5a), but not in the mobile phase (5b). Again, the two major peaks in (a) present the same UV/Vis spectrum (not shown).

As already mentioned above, the elution of double peaks in the analysis of aminoacids was reported to occur in acids or even in pure water.[3] The first peak was associated to a dimeric (or polymeric) form of the aminoacid, due to ionic or hydrogen bonding, the second one corresponding to the monomer. However, in the present case we think this seems unlikely, in a highly protic polar medium.

Without accounting the ionisation of phenolic groups, one could admit the following species in equilibrium :



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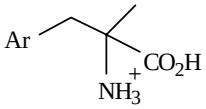

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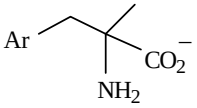
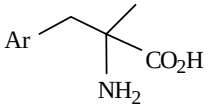


III



IV

The Different Amino Acid Ionic Forms

In organic solvents, the value of the involved equilibrium constants are quite different than in water. The specie IV, for example, which in water doesn't overtake 0.01 %, can reach as much as 10 % in DMSO.[14] In this solvent, the MD pK_{COOH} and pK_{NH_3} values are 7.3 and 8.2, respectively, against 2.2 and 10.6 taken in water.[15]

What surprised us in this case is that, although using the same mobile phase, it was verified that depending on the solvent used to prepare the injected sample (MeOH, mobile phase or aqueous H_2SO_4), one or more peaks could be eluted. This phenomenon clearly shows that homogenisation between the injected solution and the mobile phase is not so fast as one could suppose in the way that the solute seems to be solvated with the solvent primary layer long

enough into the column. So, the composition in the sample solution - in this case, the equilibrium between ionic forms - plays an important role on the eluted final mixture, although the injected amount be very small compared to the mobile phase volume passing throughout the column.

Considering the Henderson-Hasselbach equation and taking into account the carboxyl ionisation, we have:

$$pH = pK_a + \log \frac{[RCO_2^-]}{[RCO_2H]}$$

Taking the MD pK_{COOH} value as equal to 2.2[15] and being the mobile phase pH equal to 2.6, we come to:

$$\frac{[RCO_2^-]}{[RCO_2H]} = 2{,}5$$

This corresponds to 71.4 % of MD in the form I and 28.6 % in the form II. However, this calculation may not be valid for the employed mobile phase, due to the presence of methanol and the ion-pair reagent, which can change the pK_a value in a significant way. For example, the pK_a (COOH) of the own MD in a certain micellar medium was estimated in 3.3 units against the 2.2 obtained in water.[16] That is, the above relation can be inverted.

This indicates that one should be very careful in an attempt to relate retention times with molecule ionisation. We can just affirm, as a result of the present work, that initial peaks should correspond to neutral or negative species, since they are less retained when using the anionic-pairing containing system.

Finally, It is worthwhile to comment that the MD elution patterns were compared in different columns, two C_8 and another C_{18}. In both C_8-packing one peak eluted broad (Nova-Pak 150 × 3,9 mm, 5 μm part. size) or in the form of a shoulder of another peak (Shim-Pak 250 × 4,6 mm, 5 μm part. size), whereas in the other C_{18} (Lichrospher 100 125 × 4 mm, 5 μm part. size) the chromatographic behaviour was similar to those we have presented in this paper.

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