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Statistical optimization of a reversed-phase liquid chromatographic method for the analysis of amiloride and hydrochlorothiazide in tablets

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Abstract

A method has been developed for the separation of hydrochlorothiazide and amiloride by high-performance liquid chromatographic (HPLC) method on a C_{18} column with detection at 280 nm. The optimal conditions of separation were determined with the aid of 'window diagram' technique of Laub and Purnell. The effect of simultaneously varying the pH, proportion aqueous acetic acid and methanol in the mobile phase were studied to optimize the separation. A response surface diagram was used to optimize the experimental conditions for the separation. The mobile phase composition that provides an acceptable resolution hydrochlorothiazide and amiloride in a short elution time is water:methanol (60:40) and pH 3.2 (pH adjusted to 3.2 with CH_3COOH). A method is applied for the quantitative analysis of Moduretic® tablets (Merck Sharp & Dokme International). The powdered tablets are extracted with methanol, containing caffeine as the internal standard, and assayed by comparison of peak areas after liquid chromatography. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Amiloride; Hydrochlorothiazide; High-performance liquid chromatography; Internal standard; Window diagram technique

1. Introduction

Amiloride hydrochloride and hydrochlorothiazide are applied in treatment of hypertensis in tablet form. Methods for their determination in pharmaceutical formulations are based on spectrophotometry [1–4], gas–liquid chromatography (GLC) [5,6] and high-performance liquid chromatography (HPLC) [7–16].

Hydrochlorothiazide and amiloride are analyzed separately by spectrophotometry and GLC method. Numerous HPLC methods for the determination of hydrochlorothiazide [7–11], of amiloride [12–14], and both hydrochlorothiazide and amiloride [15,16] in biological fluids exist. Published HPLC methods for determination of amiloride includes fluorescence detection.

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This report describes a new, sensitive, and reproducible reversed-phase HPLC technique for the simultaneous separation and quantitation of hydrochlorothiazide and amiloride in tablets with UV detection at 280 nm.

The major goal of this investigation was to obtain quality separation of amiloride and in a reasonable analysis time by adjusting acceptable chromatographic factors. Good chromatography requires capacity factors to be neither too low (bad resolution), nor too high (long analysis time, pure detection sensitivity). A mathematical description of such goal is called an optimization criterion. Usually, the methods are based on the optimization of the mobile phase composition, i.e. on the concentration of the organic modifier and the optimization of pH. The degree of ionization of solutes, stationary phase and mobile phase additives may be affected by the pH and may lead to better selectivity. On varying the pH, the selectivity varies, but so does retention. In order to allow work at the pH value that yields the best possible selectivity, it is necessary to compensate for changes in retention. The best way is to vary the pH and aqueous/organic ratio simultaneously.

The 'window diagram' technique of Laub and Purnell [17–22] has been shown to be an effective means of locating the global optimum if a mathematical functional relationship between chromatographic retention and a single variable factor is known or can be assumed.

Single-factor systems for which the window diagram technique has been used successfully include variation of stationary phase composition in gas chromatography [17], variation of pH in liquid chromatography [22–24], and variation of lanthanide-induced shift reagent concentration in nuclear magnetic resonance spectrometry [21].

Laub and Purnell [17] have shown that plotting the relative retention (α) as a function of a single chromatographic factor (e.g. pH) for all possible pairs of compounds in a mixture gives a 'window diagram' that can be used to locate the globally optimal experimental condition. The 'windows' consist of the areas below the curves showing lowest relative retention. The experimental condition corresponding to the top of the tallest window gives the best possible separation of the two worst separated pairs of compounds [17].

This paper extends the single-factor window diagram technique to the multifactor case. Results are presented for the two-factor study in which values of pH and mobile phase composition are chosen to give the optimal chromatographic performance. The effects of methanol were examined in the range 5–50% and pH 3–6. The best set of conditions was chosen for further investigation.

2. Experimental

2.1. Equipment

Separations were made on a Waters 5 μm μ Bondapak C-18 column (300 × 3.9 mm i.d., Waters Milford, MA, USA). The injection volume was 10 μl, elution was performed at a flow rate of 1.0 ml min^{-1} and the column was maintained at ambient temperature. The absorbance was monitored at 280 nm. The mobile phase was water:methanol (60:40; v/v), pH 3.2 (adjusted with CH_3COOH)

2.2. Solvents and chemicals

Standards of amiloride and hydrochlorothiazide and Moduretic® tablets (containing amiloride hydochloride 5 mg and hydrochlorothiazide 50 mg) were supplied by Merck Sharp & Dokme International, USA. The chromatographic internal standard was caffeine. All the solvents used for the preparations of the mobile phase were HPLC grade and the mixtures were filtered and degassed before use.

2.3. Solutions

2.3.1. Internal standard solution

A 80 μg ml^{-1} solution of caffeine in methanol was prepared.

2.3.2. Stock solution

About 50 mg of hydrochlorothiazide reference material and 5 mg of amiloride reference material was precisely weighted, dissolved in internal standard solution and diluted to 50 ml. This solution (1 ml) was diluted to 10 ml with the same solvent to form a stock solution.

2.3.3. Standard solutions

Working standard solutions were prepared by dilution of a 0.7 ml volume of this solution to 10 ml with the internal standard solution. Ten solutions were prepared. The standard solutions were stable during the assay.

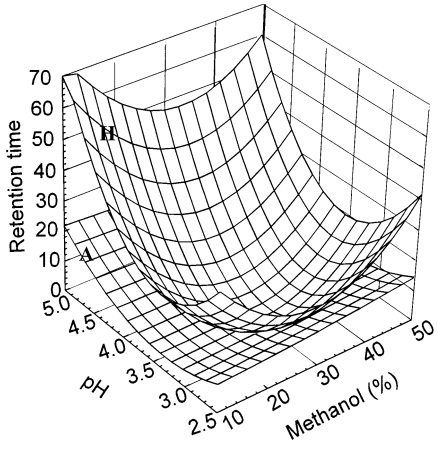

Fig. 1. Predicted retention behavior of amiloride (**A**) and hydrochlorthiazide (**H**) as function of pH and methanol ratio in mobile phase.

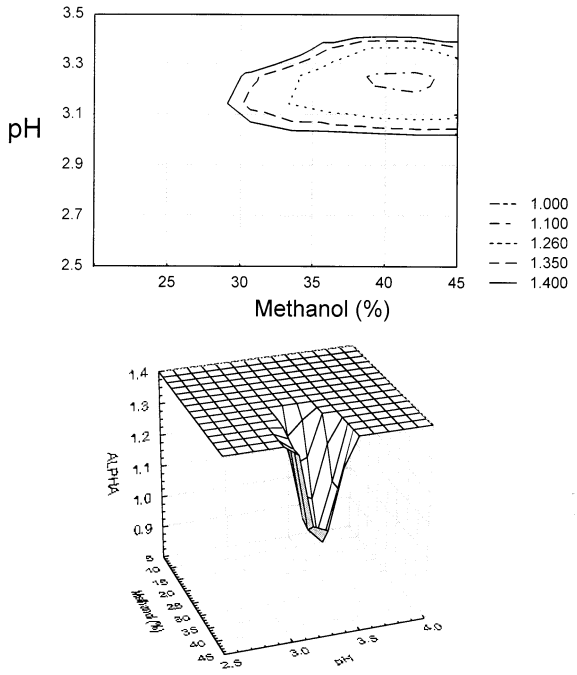

Fig. 2. Predicted relative retention (α) values for amiloride and hydrochlorothiazide as a function of pH and methanol percentage.

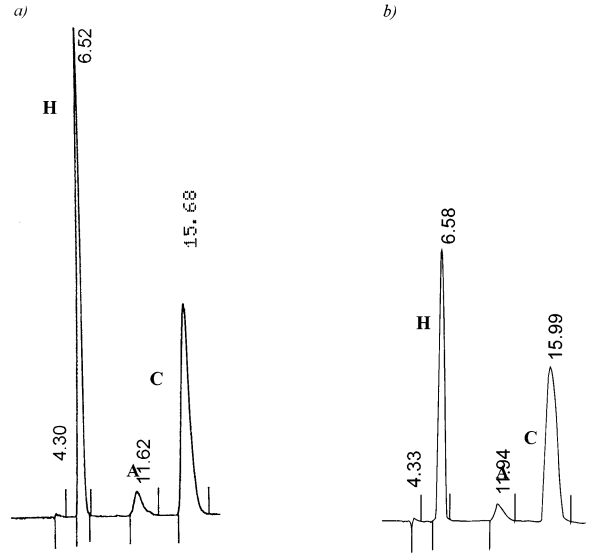

Fig. 3. (a) Separation of hydrochlorothiazide (**H**), amiloride (**A**) and caffeine as internal standard (**C**) on optimal conditions; Eluent, methanol:water (40:60); pH 3.2; flow rate, 1.5 ml min^{-1}. (b) Chromatogram of hydrochlorothiazide (**H**), amiloride (**A**) and caffeine as internal standard (**C**) in Moduretic® tablets on optimal conditions; Eluent, methanol:water (40:60); pH 3.2; flow rate, 1.5 ml min^{-1}.

Table 1
Precision of the assay expressed as % RSD of 10 samples

Sample no.	Response[a]	
	Amiloride	Hydrochlorothiazide
1	0.1105	1.050
2	0.1103	1.020
3	0.1123	1.058
4	0.1135	1.060
5	0.1105	1.045
6	0.1103	1.033
7	0.1140	1.040
8	0.1109	1.028
9	0.1104	1.050
10	0.1108	1.040
RSD	1.23	1.21

[a] Response, peak area response of drug divided by peak area of internal standard.

Table 2
Percentage recoveries obtained from 10 Moduretic® tablet sample solutions

Sample no.	Recovery (%)[a]	
	Amiloride	Hydrochlorothiazide
1	104.52	99.19
2	106.13	101.72
3	104.25	98.43
4	105.51	99.79
5	100.74	100.62
6	103.15	100.07
7	100.32	100.30
8	106.53	98.07
9	105.75	96.00
10	104.63	97.04
Mean recovery	104.15	99.13
S.D.	2.14	1.74
RSD	2.06	1.76

[a] Recovery, response of sample by response of drug.

2.4. Preparations of standard curve

Stock solutions (0.15, 0.3, 0.5, 0.9 and 1.5 ml) were accurately transferred into five 10-ml volumetric flasks and diluted to volume with the internal standard solution.

2.5. Sample preparation

A finely powdered tablet was accurately transferred to a 50 ml calibrated flask and diluted to volume with internal standard solution. The mixture was sonicated for 5 min at room temperature and than centrifuged at $2500 \times g$ for 5 min. The supernatant liquid was filtered through a 1.5 μm membrane filter. This solution (1 ml) was diluted to 10 ml with internal standard solution. A 0.7 ml volume of this solution was diluted to 10 ml with the internal standard solution. Ten solutions were prepared. The solutions of analytes were stable during the assay.

2.6. Procedure

Three injections (10 μl) of each of these solutions and of undiluted caffeine standard solution were made into the chromatographic system. The areas of the peaks were measured, and the ratios of the area peak of amiloride and hydrochlorothiazide to that of the internal standard were calculated for each injection. For calibration curve the average peak area ratio for each dilution was plotted against the quantity of amiloride and hydrochlorothiazide in the solution.

3. Results and discussion

3.1. Optimum conditions for chromatographic procedure

This work presents the results of an experimental study design to determine the combine effect of pH and mobile phase composition on the reverse-phase liquid chromatographic behavior of amiloride and hydrochlorothiazide. The effects of these factors were examined in the range of conditions where they provided acceptable retention and resolution. The effect of ratio of methanol was tested at proportion of 5–50% and effect of pH was tested at pH 3–6.

Table 3
Statistical analysis of results in the determination of amiloride and hydrochlorothiazide

($n = 10$)	Concentration (μg/ml)	Found (μg/ml)	S.D. (μg)	RSD (%)	Recovery (%)
Standard solution (bulk drug)					
Amiloride	0.7	0.7	0.01	1.23	99.5–102.8
Hydrochlorothiazide	7.0	7.0	0.08	1.28	98.9–102.8
Sample solution (Moduretic® tablets)					
Amiloride	0.7	0.729	0.01	2.06	100.3–106.53
Hydrochlorothiazide	7.0	6.94	0.12	1.79	95.94–101.7

A response surface methodology was used to specify retention time of amiloride and hydrochlorothiazide to all combination of pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and six combination of methanol:0.5% water solution of acetic acid ratio in mobile phase (5:95, 10:90, 15:85, 20:80, 30:70, 40:60).

The 'window diagram' technique pioneered by Laub and Purnell for the single factor optimization was applied to the present multifactor case to obtain optimal separation.

Fig. 1 shows the predicted retention behavior of amiloride and hydrochlorothiazide as functions of both pH and mobile phase composition. Relative retention, (α) is a better measure of separation than is the difference in retention times, as it is independent of the column efficiency [25]. The two-dimensional 'alpha diagram' shown in Fig. 2 was produced by dividing the higher capacity factor surface by the lower capacity factor surface at all combination of pH and mobile phase composition. The ratios of these capacity factor surfaces than give the relative retention surface. The domains giving acceptable separations are evident in Fig. 2 as the higher parts of the surface. Values greater than 1.4 were set equal to 14.4 [26]. The unacceptable domain occurs in the lower parts of the figure.

The mobile phase composition (of those tested) that provides acceptable resolution of amiloride and hydrochlorothiazide in a short elution time (10 min) is water:methanol (60:40) and pH is 3.2 (adjusted with CH_3COOH). There are other domains in Fig. 2 that give the same optimum results ($\alpha \cong 1.4$, quality separation within 10 min). The domain on the left is preferable because it is more rigid, and not so sensitive to the small changes in pH and methanol percentage.

Fig. 3a presents chromatogram for standard solution showing the separation under the best (optimized) conditions.

Fig. 3b presents chromatogram for Moduretic® tablets showing the separation under the best (optimized) conditions.

3.2. Quantitative determinations

The HPLC method was tested for specificity, linearity, precision and reproducibility. The specificity of the method was investigated by observing any interference between amiloride and hydrochlorothiazide and with tablet excipient. No interfering peaks and no peaks that indicate degradation products were present in the chromatograms. It is confirmed by the appearance of the baseline of chromatogram analytes (Fig. 3b) and recovery value of analytes (Table 2). The described method may use as stability-indicating assay. The k' values for hydrochlorothiazide and amiloride were 0.95 and 2.1, respectively. HPLC allows the direct analysis of amiloride in pharmaceutical dosage forms not only in the presence of the excipient, but also in formulation containing hydrochlorothiazide and vice versa. Eluting sample and standard peaks were collected and a complete ultraviolet spectrum of each peak was obtained. In all cases sample and standard peaks were found to be identical.

The linearity of the relationship between peak area and concentration was determined by analyzing five standard solutions over the concentration range 0.15–1.50 μg ml^{-1} for amiloride and 1.5–15 μg ml^{-1} for hydrochlorothiazide. The parameters of the linear regression equation were calculated for each component. The regression equation was $Y = -0.0155 + 0.1805x$ for amiloride and $Y = -0.0737 + 0.1578x$ for hydrochlorothiazide. For all analytes, the relationship between peak area ratio of drug to internal standard and concentration was highly linear over the entire concentration range (correlation coefficients of the calibration curves were greater than 0.999 and the relative standard deviation (RSD) values for the slope and the intercept with respect to the linearity were 1.5 and 1.8%, respectively, calculated at the 100% analyte level [27]. This allows only one standard solution to be used for the determination.

Limit of determination (LOD) was measured as the lowest amount of analyte that may be detected to produce a response which is significantly different from that of a blank. LOD for amiloride was 0.07 μg ml^{-1} and LOD for hydrochlorothiazide was 0.7 μg ml^{-1}.

Limit of quantification (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above baseline noise, for which

duplicate injections resulted in a RSD $\leq 3\%$. A practical LOQ giving a good precision and acceptable accuracy was 0.15 μg ml^{-1} for amiloride and 1.5 μg ml^{-1} for hydrochlorothiazide.

The precision of the chromatographic procedure was assessed by analyzing 10 solutions containing known quantities of investigated compounds. (0.7 μg ml^{-1} for amiloride and 7 μg ml^{-1} for hydrochlorothiazide). The RSD % shows the satisfactory repeatability of the system (Table 1).

Reproducibility studies were performed by analyzing 10 Moduretic® tablets (Table 2). A summary of results is presented in Table 3. Recoveries are calculated as response of sample divided by response of drug. The high recovery and the low relative standard deviation confirm the suitability of the proposed method for the routine analysis of amiloride and hydrochlorothiazide in pharmaceutical preparations.

4. Conclusions

The HPLC is efficient method for separation and quantitative determination of amiloride and hydrochlorothiazide in its dosage forms. The method provides nanogram sensitivity and adequate linearity and repeatability. There was no interference in the product examined, so no addition extraction or separation procedures are required. The method is rapid and sensitive enough to be used for single tablet analysis.

References

[1] P. Kurani, K. Desai, G. Seshadrinathan, Indian Drugs 23 (1968) 230.
[2] F. Magalhaes, G. Piros, Rev. Farm. Bioquim. Univ. Sao Paulo 8 (1970) 273.
[3] J. Vachek, Ceska Slov. Farm., Cesk. Farmacie 34 (1985) 226.
[4] M. Parissi-Poulou, V. Reizopouloov, M. Koupparis, P. Machersos, Int. J. Pharm. 51 (2) (1989) 169.
[5] A. Vondenheurerel, F. Gruber, V. Walker, J. Wlf, J. Pharm. Sci. 64 (1975) 1309.
[6] J. Lindstroen, J. Molander, J. Chromatogr. 101 (1974) 219.
[7] R. Jain, C.L. Jain, Indian Drugs 28 (8) (1991) 380–382.
[8] A. By, J.C. Ethier, G. Lauriault, M. Le Belle, B.A. Lodge, C. Savard, W.-W. Sy, W.L. Wilsan, J. Chromatogr. 469 (1989) 406–411.
[9] K.B. Alton, D. Desrivieres, J.E. Patrick, J. Chromatogr. 374 (1986) 103.
[10] G.K. Shiu, V.K. Prasad, J. Lin, W. Worsley, J. Chromatogr. 377 (1986) 430.
[11] R.M. Smith, G.A. Murilla, T.G. Hurdley, J. Chromatogr. 384 (1988) 259–278.
[12] M.S. Yip, P.E. Coates, J.J. Thiessen, J. Chromatogr. 307 (1984) 343.
[13] W.C. Vincek, G.A. Hessey, M.L. Constamzer, W.F. Bayne, Pharm. Res. 3 (1985) 143.
[14] R.J.Y. Shi, L.Z. Benet, E.T. Lin, J. Chromatogr. 377 (1986) 399.
[15] M.J. Van Der Meer, L.W. Brown, J. Chromatogr. 423 (1987) 351–357.
[16] F. De Groo, W. Van den Bossche, P. De Moerloose, Chromatographia 20 (8) (1995) 477–481.
[17] R.J. Laub, J.H. Purnell, J. Chromatogr. 112 (1975) 71.
[18] R.J. Laub, J.H. Purnell, Anal. Chem. 48 (1976) 799.
[19] R.J. Laub, J.H. Purnell, Anal. Chem. 48 (1976) 1720.
[20] R.J. Laub, J.H. Purnell, P.S. Williams, J. Chromatogr. 134 (1977) 249.
[21] R.J. Laub, A. Pelter, J.H. Purnell, Anal. Chem. 51 (1979) 1878.
[22] S.N. Deming, M.L.H. Turoff, Anal. Chem. 50 (1978) 546.
[23] W.P. Price Jr., R. Edens, D.L. Hendrix, S.N. Deming, Anal. Biochem. 93 (1979) 233.
[24] W.P. Price Jr., S.N. Deming, Anal. Chim. Acta 108 (1979) 227.
[25] P.F. Vanbel, B.L. Tilquin, P.J. Schoenmakers, Chemometr. Intell. Lab. Syst. 35 (1996) 67–86.
[26] R.C. Kong, B. Sachok, S.N. Deming, J. Chromatogr. 199 (1980) 307.
[27] G.P. Carr, J.C. Wahlich, J. Pharm. Biomed. Anal. 8 (8–12) (1990) 613–618.