Liquid Chromatography in Pharmaceutical Analysis III: Separation of Diuretic–Antihypertensive Mixtures

I. L. HONIGBERG ˣ, J. T. STEWART, A. P. SMITH, and D. W. HESTER

Abstract □ High-pressure liquid chromatography was used to optimize the resolution of therapeutic agents commonly found in antihypertensive preparations. Seven widely prescribed drugs were investigated. The compounds were chromatographed on reversed-phase octadecyltrichlorosilane (C_{18}) or diphenyldichlorosilane (phenyl) columns, using mixtures of acetonitrile or absolute methanol and distilled water buffered with ammonium acetate, ammonium chloride, or ammonium carbonate. By calculating approximate resolution values, the separation of selected drug mixtures can be predicted.

Keyphrases □ Diuretic–antihypertensive mixtures—high-pressure liquid chromatographic separation, effect of solvent composition, pH, and stationary phase polarity □ Antihypertensive–diuretic mixtures—high-pressure liquid chromatographic separation, effect of solvent composition, pH, and stationary phase polarity □ Multicomponent dosage forms—high-pressure liquid chromatographic separation of diuretic–antihypertensive mixtures □ High-pressure liquid chromatography—separation, diuretic–antihypertensive mixtures

High-pressure liquid chromatography (HPLC) has been successfully applied to the analysis of multicomponent pharmaceutical dosage forms in this laboratory (1, 2). In a continuation of the study of multicomponent dosage forms, this paper reports an investigation of the parameters associated with the separation and detection of diuretic–antihypertensive agents.

The compounds studied are included in listings of most prescribed drugs used singly or in combination for the treatment of hypertension. The drugs are chlorothiazide, hydrochlorothiazide, triamterene, hydralazine hydrochloride, guanethidine sulfate, methyldopa, and reserpine. A preliminary report on the sequential quantitative analysis of a chlorothiazide–reserpine mixture was published (2).

Chlorothiazide and hydrochlorothiazide in single entity and combination dosage forms have been analyzed by many methods including absorption spectroscopy (3–5), colorimetry (6), amperometry (7), bromatometric and bromo–chlorometric titrimetry (8), complexometric titrimetry (9), and nonaqueous titrimetry (10, 11). Triamterene can be measured by absorption spectroscopy, fluorescence, and TLC fluorometry (12–14), while hydralazine and guanethidine have been assayed by various titrimetric methods (15, 16). Fluorescence methods have been utilized for the determination of methyldopa (17) and reserpine (3, 18). Other procedures reported for these latter drugs include polarography (19) and colorimetry (20, 21).

The HPLC operating parameters investigated were limited to solvent composition, pH, and stationary phase polarity. The use of these data can be readily applied to the separation of selected multidrug dosage forms. This use has been exemplified by the separation and quantification of chlorothiazide–reserpine at unit-dosage levels (2).

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of chlorothiazide[2], hydrochlorothiazide[2], triamterene[3], hydralazine hydrochloride[4], guanethidine sulfate[4], methyldopa[2], and reserpine[4] were used in the analytical procedure. All other chemicals and solvents were the highest grade commercially available.

Mobile Phases—The mobile phases consisted of various concentrations of acetonitrile or absolute methanol mixed with aqueous solutions of 0.1 or 1% ammonium acetate, 0.5 or 1% ammonium chloride, and 0.2 or 1% ammonium carbonate. The solutions were prepared fresh daily.

Drug Solutions—Each drug was prepared as a 0.5% solution using the appropriate mobile phase.

Chromatographic Separation—The degassed mobile phase was pumped through columns containing either monomolecular layers of octadecyltrichlorosilane (C_{18}) or diphenyldichlorosilane (phenyl) chemically bonded to a high-efficiency pellicular packing, consisting of solid glass cores with a porous silica surface[5]. A flow rate of 1.4 ml/min (1000–2000 psig) was used until a stable baseline was obtained. The column and solvents were kept at ambient temperature. Replicate 20-μl injections of the sample solutions were made using a 25-μl syringe[6]. The chart recorder provided a record of the elution of the drugs from the column as peaks on a chromatogram.

RESULTS AND DISCUSSION

The purpose of this study was to determine the operating conditions for HPLC that would optimize the resolution of chemically unrelated therapeutic agents most commonly found in antihypertensive preparations. Three operating parameters were studied: *(a)* variations in solvent composition containing acetonitrile or absolute methanol with changing concentrations of distilled water; *(b)* variations in pH within the 5.5–9.0 range using ammonium acetate, ammonium chloride, or ammonium carbonate as inorganic buffers; and *(c)* variations in the nonpolar (reversed phase) stationary phase in which the pellicular packing differed in polarity.

The effects of these parameters on retention times and peak widths of the drugs are shown in Table I. In addition, multiple entries in Table I indicate those solvent systems (Table II) that generate multiplets for a component. These multiplets may arise from decomposition of the drug and/or the varied species of the drug possible at the mobile phase pH used. Significant tailing of single-drug entities is also noted.

The following generalizations can be inferred from the data in Table I. Retention time for many of the drugs increases as the polarity of the mobile phase increases. This generalization can be obscured when the solute travels as a multiple species such as methyldopa. It is also difficult to observe this effect for drugs that travel close to the void volume of the column and for which it is difficult to measure small differences in retention time. This situation is exemplified by chlorothiazide and hydrochlorothiazide. When

[1] A Waters Associates liquid chromotograph (model ALC 202), equipped with a M-6000 pump, UV (254 nm) and differential refractive index monitors, and Waters packed columns, 1.22 m long × 2.3 mm i.d., was used.

[2] Merck Sharp and Dohme, Rahway, N.J.

[3] Smith Kline and French Labs., Philadelphia, Pa.

[4] Ciba, Summit, N.J.

[5] Corasil/C_{18} and Corasil/phenyl, 37–50 μm, Waters Associates, Milford, Mass.

[6] Model B-110, Precision Sampling Corp., Baton Rouge, La.

Table I—Effect of Mobile Phase Composition on Retention Times[a]

Mobile Phase Composition[b]	Chlorothiazide		Hydrochlorothiazide		Triamterene		Hydralazine		Guanethidine[c]		Methyldopa		Reserpine	
	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl
A		94[d] (26)[f]		95 (33)		107 (28)		131 (64)		372[e] (137)		102 (37) 136 (56)		99 (25)
B		101 (24)		115 (32)		105 (30)		159[e] (222)		449[e] (204)		116[e] (93)		130 (42)
C	90 (20)		95 (24)		108 (20)		102 (52) 141 (44) 333[e] (167)		—[g]		94 (30)		192 (49)	
D		95 (22)		100 (32)		123 (27)		109 (44) 132 (50) 168 (90)		425[e] (123)		105 (63)		219 (66)
E		99 (19)		108 (26)		154 (38)		220 (79)		587[e] (231)		125[e] (105)		—[g]
F	96 (20)	102 (23)	96 (24)	102 (25)	145 (44)	129 (36)	224 (155)	108 (20) 153 (128) 261[e] (715)	185 (67)	103 (39) 483[e] (233)	109 (37) 140 (63)	115 (33) 139 (30) 169 (240)	1950 (800)	1440[e] (1000)
G	92 (22)	92 (18)	92 (27)	92 (22)	100 (24)	101 (39)	123 (92)	98 (23) 112 (43) 135 (46)	125 (37) 153 (56)	150 (35) 243 (57)	98 (82)	95 (24)	98 (23)	99 (20)
H	95 (20)	92 (21)	95 (25)	95 (25)	100 (24)	101 (27)	137[e] (240)	142 (115) 172 (130)	94 (18) 122 (38)	95 (24) 119 (25)	93 (20)	117[e] (138)	138 (35)	120 (30)
I		98 (24)		100 (24)		145 (70)		128 (80) 508[e] (250)		90 (27) 141 (57)		139[e] (140)		—[g]
J	99 (20)	102 (25)	99 (25)	101 (27)	126 (45)	124 (40)	140 (80)	188[e] (100)	99 (27) 180 (96)	102 (30) 299[e] (160)	102 (28)	109 (27)	374[e] (168)	224 (138)

K	113 (25)		108 (37)		271[e] (152)		306[e] (133)		108[e] (30) 435[e] (172)			118 (40)							—[g]	
L	82 (20)		90 (27)		98 (21)		106 (31) 192 (53)		—[g]		91 (22) 115[e] (92)				148 (37) 214 (62)					
M	98 (20)		97 (26)	101 (24)	105 (25)	137 (43)	121 (30)	106 (26) 126[e] (81)	135 (60) 169 (73)	—[g]		101 (30) 143[e] (73)		99 (22) 125 (37) 235[e] (298)	104 (75) —[g]				132 (44) 1047 (920)	
N		98 (20)		105 (24)			119 (27)		113 (36) 367[e] (443)	—[g]			104 (45) 783[e] (960)						107 (40) 621 (580)	

[a] Time elapsed between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using UV detection unless otherwise noted. [b] Solvent compositions and pH are given in Table II. [c] The eluted peaks were monitored using a differential refractive index detector. [d] Retention time expressed as seconds. Variability is ±12 sec. [e] Significant tailing was observed. [f] Peak width expressed as seconds. [g] Retention time greater than 2400 sec.

Table II—Solvent Compositions and pH

Mobile Phase	Components	Solvent Ratio	pH
A	Acetonitrile	80	7.35
	0.1% Ammonium acetate	20	
B	Acetonitrile	60	7.35
	0.1% Ammonium acetate	40	
C	Acetonitrile	50	7.35
	0.1% Ammonium acetate	50	
D	Acetonitrile	40	7.35
	0.1% Ammonium acetate	60	
E	Acetonitrile	20	7.35
	0.1% Ammonium acetate	80	
F	Absolute methanol	50	7.10
	1% Ammonium acetate	50	
G	Acetonitrile	80	6.10
	1% Ammonium chloride	20	
H	Acetonitrile	50	5.57
	1% Ammonium chloride	50	
I	Acetonitrile	20	5.56
	1% Ammonium chloride	80	
J	Absolute methanol	50	5.60
	0.5% Ammonium chloride	50	
K	Absolute methanol	20	5.60
	0.5% Ammonium chloride	80	
L	Acetonitrile	50	8.80
	0.2% Ammonium carbonate	50	
M	Absolute methanol	50	8.45
	0.2% Ammonium carbonate	50	
N	Absolute methanol	50	8.70
	1% Ammonium carbonate	50	

the pH of the mobile phase is adjusted so that the unionized species of the drug predominates, as calculated from its pKa value (15, 22, 23)[7], an increased retention time is noted for many of the drugs. Guanethidine, methyldopa, and triamterene are exceptions to this effect. A comparison of solvents containing equivalent amounts of acetonitrile–water and methanol–water showed that retention times are greater in the methanol-containing solvents. This finding is consistent with the observation of the effect of mobile phase polarity on retention time. The interplay of mobile phase pH and polarity and their effects on either the ionizability of the traveling solute or the ability to solvate the solute may evert the outlined generalizations.

Modifications in the polarity of stationary and mobile phases provided the following observations for the drugs examined. Chlorothiazide and hydrochlorothiazide traveled close to the solvent front in all systems. Triamterene could be separated from the thiazide diuretics in Solvent Systems E and K (Table III)[8]. The remaining solvent systems, such as exemplified by mobile phases I and N, did not adequately resolve these compounds. The drug was resolved best using the more polar mobile phases.

Hydralazine gave multiplets in most solvents. The exceptions were Solvents A, B, E, J, and K. Solvents A and E gave symmetrical peaks while B, J, and K showed tailing.

The aliphatic character of guanethidine precluded its detectability by UV absorption. Thus, differential refractometry was used to monitor the column effluent. Sensitivity was in the range of 50–100 μg for guanethidine. Acetonitrile–water mixtures were most effective in producing single symmetrical peaks for guanethidine at pH 7.35. However, as the pH of the mobile phase was made acidic, a doublet was observed.

Methyldopa was the most difficult to resolve from the other drugs in this study. In systems where the retention time of methyldopa was increased by the addition of water, excessive tailing was obtained. In mobile phases with pH >8, multiple peaks were obtained indicative of the several species of methyldopa possible in basic media. At pH <7, no multiplets were observed. In solvent systems where methyldopa eluted as a single peak, it could not be

[7] Guanethidine pKa value supplied by Dr. J. Mollica, Ciba-Geigy, Suffern, N.Y., personal communication, 1974.

[8] It is possible to calculate the approximate resolution *(Rs)* of two components by the equation: $Rs = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times, and w_1 and w_2 are base peak widths of Compounds 1 and 2, respectively. Experience indicates that two components with an *Rs* value >1.5 show satisfactory resolution by this analytical technique.

Table III—Calculation of Approximate Resolution (*Rs*)[a] Values for Triamterene—Thiazide Mixtures

Drug Mixture	Mobile Phase[b] E	K	I	N
Triamterene	154[c]	271	145	119
	(38)[d]	(152)	(70)	(27)
Chlorothiazide	99	113	98	98
	(19)	(25)	(24)	(20)
Rs =	1.9	1.8	1.0	0.89
Triamterene	154	271	145	119
	(38)	(152)	(70)	(27)
Hydrochlorothiazide	108	108	100	105
	(26)	(37)	(24)	(24)
Rs =	1.4	1.7	0.96	0.55

[a] See footnote 8. [b] Letters refer to solvent compositions in Table II. [c] Retention time on phenyl column from Table I. [d] Peak width on phenyl column from Table I.

Table IV—Calculation of Approximate Resolution (*Rs*)[a] Values for Hydrochlorothiazide—Hydralazine—Reserpine Mixture

Drug Mixture	Mobile Phase[b] E	F	K
Hydrochlorothiazide	108[c]	102	108
	(26)[d]	(25)	(37)
Hydralazine	220	224	306
	(79)	(155)	(133)
Rs =	1.95	1.36	2.3
Hydralazine	220	224	306
	(79)	(155)	(133)
Reserpine	>2400	1950	>2400
		(800)	
Rs =	>6.0[e]	3.6	>5.7[e]

[a] See footnote 8. [b] Letters refer to solvent compositions in Table II. [c] Retention time data from Table I. [d] Peak width data from Table I. [e] Calculated value based on retention time of 2400 sec and peak width of 800 sec for reserpine.

resolved from the thiazides. However, solvents systems such as D, J, and K could resolve methyldopa from reserpine; D and K could resolve methyldopa from both guanethidine and reserpine.

At high concentrations of acetonitrile, reserpine traveled with the solvent front. Its retention time was increased as the polarity of the solvent increased. In many instances, the retention time of reserpine was much greater than that of the remaining drugs tested and resulted in considerable band spreading. However, the reserpine peaks were symmetrical and would be suitable for quantification. In solvent systems with pH >8, several additional peaks were detected in addition to the reserpine band. This finding may be due to rapid decomposition of reserpine in alkaline media (24). Table IV illustrates resolution values for the three-component mixture of hydrochlorothiazide, hydralazine, and reserpine in some mobile phases. Solvent Systems E and K seem most suitable for separating the mixture, but E is better since hydralazine exhibits considerable tailing in mobile phase K. A flow programming procedure (changing flow rate with time) would be necessary with Solvent System E to decrease the retention time for reserpine.

Information contained in Table I presents operating parameters for HPLC that can be used to optimize the resolution of the multifarious drugs found in antihypertensive dosage forms. With the calculation of approximate *Rs* values as described previously, the separation of selected drug mixtures can be predicted.

REFERENCES

(1) I. L. Honigberg, J. T. Stewart, and A. P. Smith, *J. Pharm. Sci.*, **63,** 766(1974).

(2) I. L. Honigberg, J. T. Stewart, A. P. Smith, R. D. Plunkett, and D. W. Hester, *ibid.*, **63,** 1762(1974).

(3) T. Urbanyi and A. O'Connell, *Anal. Chem.*, **44,** 565(1972).

(4) F. Fazzari, *J. Ass. Offic. Anal. Chem.*, **55,** 161(1972).

(5) R. Chu, *ibid.*, **54,** 603(1971).

(6) J. Perez, A. Danhier, and J. Brieva, *Ann. Real. Acad. Farm.*, **33,** 233(1967); through *Chem. Abstr.*, **68,** 98681(1968).

(7) M. Madgearu, H. Beral, and E. Cuciureanu, *Farmacia*, **16,** 471(1968); through *Chem. Abstr.*, **70,** 6558(1969).

(8) P. Kertesz, *Acta Pharm. Hung.*, **39,** 127(1969); through *Chem. Abstr.*, **71,** 33474(1969).

(9) N. Teodorescu, P. Laurentia, and G. Ciogolea, *Farmacia*, **15,** 13(1967); through *Chem. Abstr.*, **66,** 98546(1967).

(10) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 160.

(11) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 302.

(12) L. Guerello and J. Dobrecky, *Rev. Farm.*, **111,** 13(1969); through *Chem. Abstr.*, **71,** 64116(1969).

(13) E. Densivil and J. Vismans, *Pharm. Weekbl.*, **105,** 1441(1970).

(14) W. Bruehl and E. Schmid, *Arzneim.-Forsch.*, **20,** 485(1970).

(15) K. Erstratova and A. Ivanova, *Farmatsiya (Moscow)*, **17,** 41(1969); through *Chem. Abstr.*, **69,** 46128(1968).

(16) I. Simonyi and J. Varga, *Acta Pharm. Hung.*, **41,** 78(1971); through *Chem. Abstr.*, **74,** 130443(1971).

(17) R. Laverty and K. Taylor, *Anal. Biochem.*, **22,** 269(1968).

(18) H. Stober and T. Urbanyi, *J. Ass. Offic. Anal. Chem.*, **55,** 180(1972).

(19) J. T. Stewart, H. C. Loo, and W. D. Mason, *J. Pharm. Sci.*, **63,** 954(1974).

(20) C. R. Szalkowski and W. J. Mader, *J. Amer. Pharm. Ass., Sci. Ed.*, **45,** 613(1956).

(21) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 587.

(22) W. F. Charnicki, F. A. Bacher, S. A. Freeman, and D. H. DeCesare, *J. Amer. Pharm. Ass., Sci Ed.*, **48,** 656(1959).

(23) "Perspectives in Clinical Pharmacy," D. E. Franke and H. A. K. Witney, Eds., Drug Intelligence Publications, Hamilton, Ill., 1972, p. 334.

(24) O. Weis-Fogh, *Pharm. Acta Helv.*, **35,** 442(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1974, from the *Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30602*

Accepted for publication December 24, 1974.

[x] To whom inquiries should be directed.