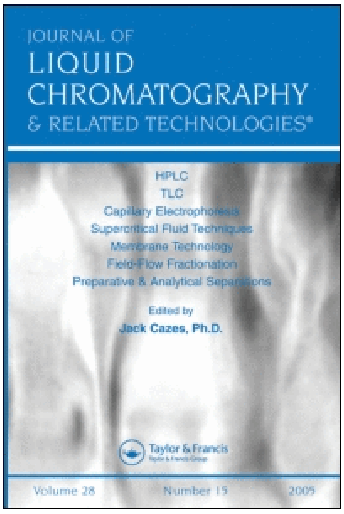

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Kenley, R. A. , Partridge, I. W. and Fleitman, J. S.(1986) 'Diastereospecific HPLC Analysis of the Antiulcer Drug, Enprostil, in a Soft Elastic Gelatin Capsule Formulation', Journal of Liquid Chromatography & Related Technologies, 9: 11, 2457 — 2470

To link to this Article: DOI: 10.1080/01483918608076878

URL: http://dx.doi.org/10.1080/01483918608076878

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(11), 2457-2470 (1986)

DIASTEREOSPECIFIC HPLC ANALYSIS OF THE ANTIULCER DRUG, ENPROSTIL, IN A SOFT ELASTIC GELATIN CAPSULE FORMULATION

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ABSTRACT

Enprostil, an E-type prostaglandin analog, is formulated as a 0.3 mM propylene carbonate solution filled into soft elastic gelatin capsules. A characteristic structural feature of enprostil is the presence of two unresolved chiral centers, yielding a drug molecule that exists as two diastereomeric pairs of enantiomers. This report describes an HPLC method for quantitatively determining the relative amounts of enprostil diastereomers in soft elastic gelatin capsules. Using a Spherisorb ODS 5 µm, 250 x 4.5 mm column and a mobile phase mixture of 59:40:1 methanol:water:tetrahydrofuran separates the enprostil diastereomeric pairs and also resolves the enprostil diastereomers from potential interferences arising from placebo and drug degradation. The method performs well as evidenced by the usual statistical tests for: response linearity, recovery efficiency, precision, and lower quantitation limit. Also, the demonstrated dependence of chromatographic retention on mobile phase composition and system operating parameters defines acceptable operating condition ranges.

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0148-3919/86/0911-2457$3.50/0

INTRODUCTION

Our previous communication (1) describes a quantitative analysis for the antiulcer prostaglandin, enprostil, in a soft elastic gelatin capsule formulation. Although the previously-described analytical method satisfactorily determines low concentrations of enprostil, the method is not specific for enprostil stereoisomers. Figure 1 shows the the four isomers provided by the current enprostil synthesis. The unresolved chiral centers at C5 and C8 give two diastereomeric pairs of enantiomers. According to the shorthand nomenclature shown in Figure 1, the A1 and A2 isomers are related as mirror images (enantiomers) as are the B1,B2 isomers whereas the A1,B2 and A2,B1 pairs are diastereomeric.

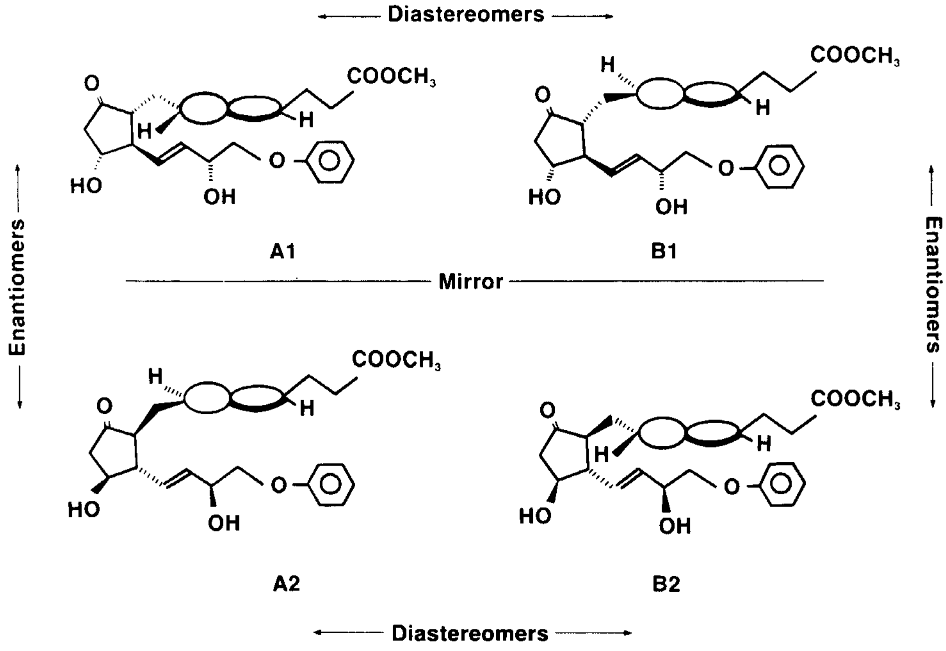

Figure 1. Structural Representation and Shorthand Nomenclature for Enprostil Stereoisomers.

Diastereomers can, in principle, exhibit different chemical reactivity and physical properties. This principle raises the possibilities that: 1) in soft elastic gelatin capsules, the enprostil diastereomer ratio depends initially on manufacturing variables, or 2) the diastereomer ratio changes with time during storage. To directly demonstrate the degree to which enprostil diastereomer ratios are independent of manufacturing procedures or environmental conditions required a diastereospecific assay. Accordingly we modified the previously-described method to resolve the A1,A2 and B1,B2 enprostil isomers. The following sections detail: procedures for the diastereospecific assay method, statistical tests of method performance, and tests of method sensitivity to operating conditions.

EXPERIMENTAL DETAILS

Materials. Enprostil and authentic samples of the individual isomers, A1 and B1 were prepared by the Syntex Institute of Organic Chemistry. Chemical structures and purities of these compounds were established by the usual spectroscopic and elemental analyses. Optical purities of the A1 and B1 isomers were assessed by determining specific rotation and by circular dichroic spectra. Thus, A1 gave $[\alpha]_D$ (5 mg/mL in methanol) = -3.0 and $[\theta]_{max}$ (0.25 mg/mL in acetonitrile) = -11700 at 301 nm. B1 gave $[\alpha]_D$ = -91.9 and $[\theta]_{max}$ = -14300 under the same conditions. Other materials were as previously described (1).

Instrumentation. The diastereospecific analysis employed a Spherisorb ODS 5 µm, 250 x 4.5 mm column. The following components comprised the HPLC system: model SP8770 pump

(Spectra-Physics), model 725 automatic injector (Micromeritics), model SP8440 detector (Spectra-Physics), model 748 C column oven (Spectra-Physics), model SP4100 integrator (Spectra-Physics).

Operating Conditions. The mobile phase was a 59:40:1 mixture of methanol, water, and THF. The flow rate was set to 1.8 mL/min and the column oven maintained at 25° +/- 2°C. Under these conditions column pressures ranged from 3800 to 4600 psi. With 100 µL injected (= 1.25 µg enprostil) and 220 nm detection, the detector output range was set to 0.02 AUFS.

Sample Preparation and Assay. Five capsules were cut with scissors and the contents combined in a scintillation vial. A 0.5-mL aliquot was removed with a to-contain pipette and diluted to 5 mL with a 60:40 mixture of water and methanol. The latter solution was filtered through a Nylon 66 membrane filter and directly injected. Total enprostil concentrations were determined using the previously described method and the results reported as a percentage of the dosage form labeled strength (= 0.3 mM). Diastereomer ratios were calculated as the fraction of A1 plus A2 isomers or B1 plus B2 isomers present, that is, as shown in equation (1) and (2):

$$F_A = ([A1] + [A2])/([A1] + [A2] + [B1] + [B2]) \qquad (1)$$

$$F_B = ([B1] + [B2])/([A1] + [A2] + [B1] + [B2]) \qquad (2)$$

Statistical Validation. To test for method response linearity and recovery, eleven placebos were spiked with enprostil and with A1 and B1 isomers to give final solutions containing $F_B = 0.25$ to 0.75 and total enprostil = 100 to 150% of

0.3 mM. The spiked placebos were then assayed in duplicate and the results for recovery and linearity calculated according to equations (3) and (4), respectively:

$$\% \text{ Recovery} = 100*[\% \text{ Isomer}]_{\text{found}}/[\% \text{ Isomer}]_{\text{added}} \tag{3}$$

$$[\% \text{ Isomer}]_{\text{found}} = \text{Intercept} + \text{Slope}*[\% \text{ Isomer}]_{\text{added}} \tag{4}$$

where [% Isomer] represents the percentage of A1 plus A2 or B1 plus B2 present as calculated by equation (5):

$$[\% \text{ Isomer}] = \{100*[\text{total enprostil, mM}]*[F_A \text{ or } F_B]\}/(0.3 \text{ mM}) \tag{5}$$

To test method precision, two analysts on two different days assayed twelve capsule samples. To assess the method lower quantitation limit, placebos were spiked at 2 to 30% of 0.3 mM with enprostil and [% Isomer] values determined according to equation (5). From the the independently-measured diastereomer ratio of the spiking solution ([% B] = 48.7 with +/- 95% confidence limits = 0.9%) the [% Isomer] values in the spiked placebo were calculated and compared with the observed values. The lower quantitation limit was then established as the lowest concentration of added enprostil giving observed isomer percentages within the 95% confidence limits for the calculated values (see Results and Discussion Section for further description).

System Suitability. To test the degree to which system operating parameters influence chromatographic performance, we prepared a methanolic solution of enprostil and obtained chromatograms using different: Spherisorb ODS 5 µm columns, mobile phase compositions, flow rates , and temperatures. For each chromatogram we calculated capacity factor (k') values, theoretical plate counts (N), tailing factor (T_s) at 10% of peak height, and resolution (R) between the diastereomers. The calculations employed the usual equations (2).

RESULTS AND DISCUSSION

Representative Chromatograms. Figure 2 shows representative chromatograms for placebo, standard solution and a soft elastic gelatin capsule sample. The B1,B2 enantiomers coeluted as the first major peak, and the diastereomeric A1,A2 isomers coeluted in the subsequent major peak. The chromatogram shows partial resolution between the isomeric pairs -- no set of operating conditions investigated achieved baseline resolution between the A and B diastereomers. As described below, however, the resolution typically observed with the selected operating parameters permitted accurate quantitation of the isomer fractions F_A and F_B. Method specificity was demonstrated by coinjecting enprostil with authentic samples of known or potential interfering compounds (see reference 1 for a description of the potential interferences). For conciseness, the chromatograms are not shown here.

Statistical Tests of Method Performance. Table I summarizes the statistics that characterize the method performance under the specified operating conditions. Despite the incomplete resolution observed in representative chromatograms (Figure 1), the method proved to be highly accurate, linear, and precise. Table II provides calculated versus observed [% Isomer] values for placebos spiked with 2 to 30% of 0.3 mM enprostil. Twelve replicate injections of the spiking solution gave the "calculated" isomer fractions, F_A = 0.513 +/- 0.0090 and F_B = 0.487 +/ 0.0090, where the error limits are expressed as

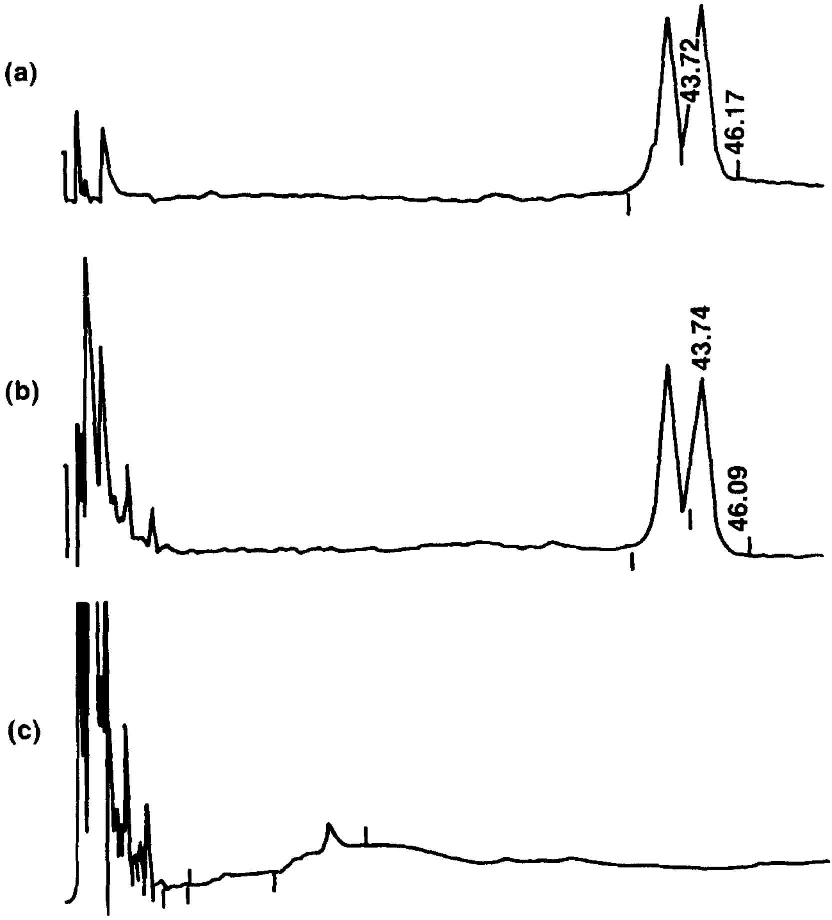

Figure 2. Representative Chromatograms of: (a) Enprostil Standard Solution, (b) Enprostil in SEG capsule, and (c) Placebo Capsule. Retention times shown in minutes. The first peak contains the B1,B2 enantiomers and the later peak contains the diastereomeric A1,A2 pair of enantiomers.

TABLE I. STATISTICAL INDICATORS[a] OF METHOD PERFORMANCE FOR DIASTEREOSPECIFIC ANALYSIS OF ENPROSTIL

Indicator	Statistic	Units	A1,A2 Peak	B1,B2 Peak	Equation
Recovery Efficiency	Mean	%	99.8	100	3
	Standard Deviation	-	0.70	0.90	
	Coefficient of Variation	-	0.70	0.90	
Response Linearity	Slope (± 95% Conf. Inter.)	-	0.998 (0.010)	0.993 (0.012)	4
	Intercept (± 95% Conf. Inter.)	%	-0.021 (0.52)	0.358 (0.62)	
	Correlation Coefficient	-	0.9998	0.9997	
	Standard Error of Regression	-	0.339	0.417	
Precision	Mean	[% Isomer][c]	51.1	48.9	b
	Standard Deviation	[% Isomer][c]	0.22	0.22	
	Coefficient of Variation	-	0.42	0.44	

[a] See experimental section for details of validation tests.
[b] See text.
[c] See equation (5).

TABLE II. OBSERVED VERSUS CALCULATED [% ISOMER] VALUES FOR DIASTEREOSPECIFIC ASSAY OF ENPROSTIL SPIKED PLACEBOS

Enprostil Added % of 0.3 mM	[% B Isomers] Found	[% A Isomers] Found	[% B Isomers] Calculated[a] ± 95% C.I.	[% A Isomers] Calculated[a] ± 95% C.I.
30	48.8	51.2	48.7 ± 0.9	51.3 ± 0.9
25	49.3	50.7	"	"
20	49.1	50.9	"	"
12.3	50.0	50.0	"	"
9.9	46.4	53.6	"	"
4.9	46.1	53.9	"	"
2.0	42.9	57.1	48.7 ± 1.0	51.3 ± 1.0

[a] See text.

95% confidence intervals about the mean. Comparing observed versus calculated [% Isomer] values in Table II shows that spiked placebos containing less than 20% total enprostil gave observed [% Isomer] values outside the confidence intervals for the calculated values. Thus, we assign 20% of 0.3 mM as the lower quantitation limit for the method.

Influence of System Parameters on Method Performance. Table III summarizes the results of system suitability tests for the diastereospecific assay. The first three rows in Table III show the column-to-column variation in chromatographic performance. We investigated several reverse-phase columns from different manufacturers and found the Spherisorb ODS 5 µm , 250 x 4.6 mm i.d. column to be the only suitable column for the analysis. Even for this column type, individual columns afforded quite different separations. For all three of the tested Spherisorb ODS columns, satisfactory resolution obtained, but total plate counts ranged from 2500 to over 8000 (with respect to the A1,A2 pair) and

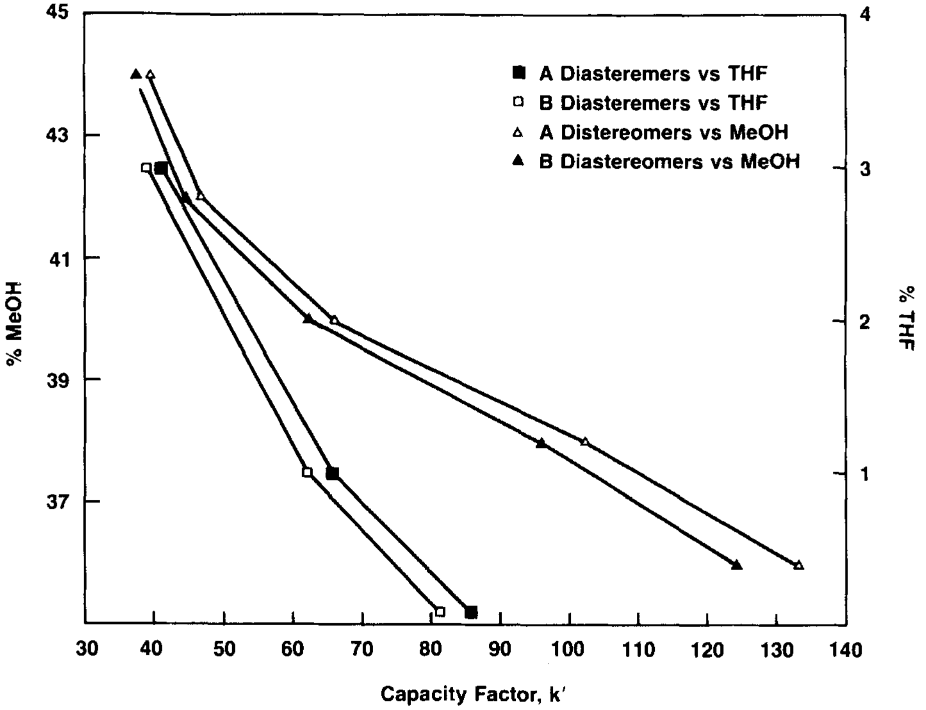

Figure 3. Mobile Phase Composition Influence on Retention of Enprostil A1,A2 and B1,B2 Diastereomers.

resolution factors ranged from 0.66 to 1.2. The observed method performance under these ranges allows us to establish minimum performance criteria for suitability of the system. Thus, to accurately quantitate enprostil diastereomer ratios, total plate count should exceed 2500, the resolution factor should exceed 0.66 and the tailing factor value should range between 0.8 to 1.3.

Table III also shows the chromatography to be very sensitive to mobile phase composition, flow, and temperature. Figure 3 shows the dependence of enprostil diastereomer

TABLE III. CHROMATOGRAPHIC PERFORMANCE PARAMETERS[a] FOR DIASTEREOSPECIFIC ANALYSIS OF ENPROSTIL[b]

Column[c] #	THF[d] Fraction	MeOH[d] Fraction	Flow Rate mL/min	Temp.	k' B1,B2	k' A1,A2	R	N B1,B2	N A1,A2	T B1,B2	T A1,A2
56	1	40	1.8	Ambient	50.0	52.8	1.08	6700	5900	1.20	1.10
64	1	40	1.8	Ambient	20.5	21.6	0.66	2700	2500	1.57	0.86
112	1	40	1.8	Ambient	62.1	65.7	1.2	7800	8200	1.36	1.32
112	1	44	1.8	Ambient	36.8	38.8	1.01	6460	6500	0.87	0.97
112	1	42	1.8	Ambient	44.2	46.6	1.06	7300	7000	0.92	0.95
112	1	38	1.8	Ambient	95.7	102.2	1.27	8600	8400	1.07	1.03
112	1	36	1.8	Ambient	125.	133	1.37	8300	8000	1.00	1.07
112	0	41	1.8	Ambient	81.2	85.6	1.29	8800	10300	0.97	1.02
112	3	38	1.8	Ambient	38.6	40.7	1.11	6900	6800	0.78	0.65
112	1	40	1.5	Ambient	67.8	71.7	1.25	8000	8700	1.13	1.09
112	1	40	1.8	40°C	30.5	31.7	0.79	7000	6600	1.19	1.38

[a] See text for parameter definition.

[b] Recommended conditions: mobile phase 59:40:1 mixture of H_2O, MeOH, THF; flow = 1.8 mL/min, temp = ambient.

[c] Spherisorb ODS 5, 5 μm, 250 x 4.6 mm column.

[d] Remainder = water.

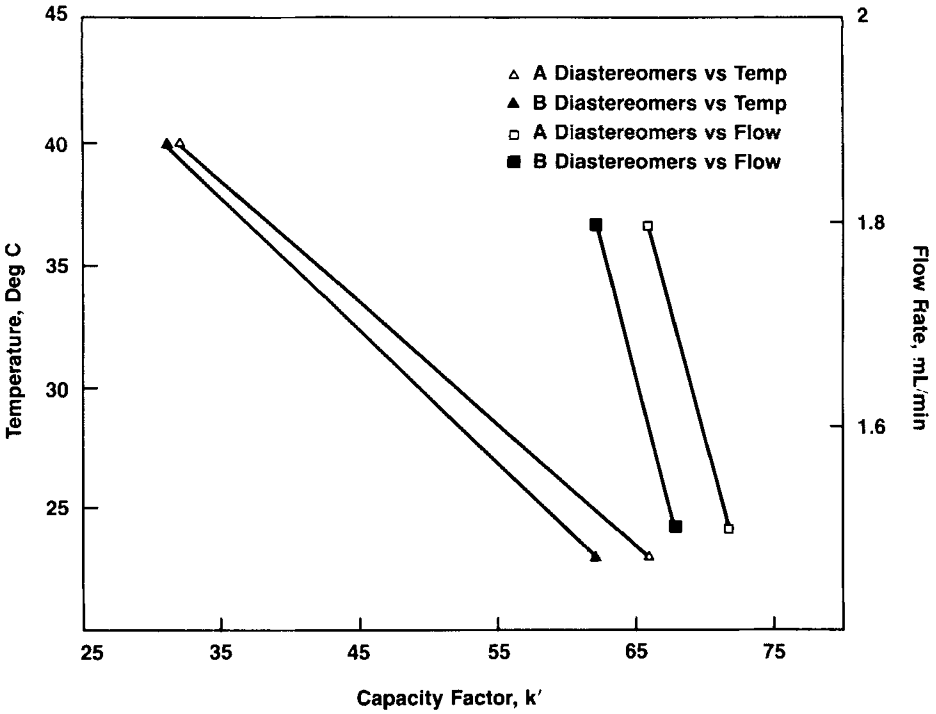

Figure 4. Column Temperature and Mobile Phase Flow Rate Influence on Retention of Enprostil A1,A2 and B1,B2 Diastereomers.

TABLE IV. TIME DEPENDENCE OF ENPROSTIL DIASTEREOMER RATIO IN THREE DIFFERENT LOTS OF SOFT ELASTIC GELATIN CAPSULES MAINTAINED AT VARIOUS TEMPERATURES

	Storage Temp.	[% B Isomer]:[% A Isomer] at Time (mo) =			
Lot #	°C	4	6	9	12
1	5				49.9:50.1
	25				49.8:50.2
	40	50.0:50.0	49.7:50.3	49.9:50.1	
2	5				49.7:50.3
	25				49.6:50.4
	40	49.7:50.3	49.8:50.2	49.6:50.4	
3	5			47.3:52.7	
	25			47.4:52.6	
	40	47.2:52.8	47.2:52.8		

capacity factor (k') values on mobile phase methanol fraction (at constant THF fraction) and on THF fraction (at constant organic:aqueous fraction). Similarly, Figure 4 plots isomer k' values versus mobile phase flow rate and temperature. Although the enprostil diastereomers resolved under all tested conditions, Figures 3 and 4 demonstrate that retention drastically decreased with increasing methanol and THF content, and with increased temperature and flow rate. Generally the figures show that selecting operating conditions to achieve shorter run times, lower column pressure, etc. has the disadvantage of decreasing resolution. The operating parameters reported here strike a working balance between method convenience and performance.

Capsule Sample Assays. To demonstrate the independence of enprostil diastereomer ratios on soft elastic gelatin capsule manufacturing variables and on storage conditions, we prepared three individual lots, maintained them at 5, 25, and 40°C, and withdrew samples for analysis at timed intervals. Table IV lists the results and shows conclusively that (within the limits of experimental uncertainty) enprostil soft elastic gelatin capsule diastereomer ratios were constant from lot to lot and invariant with time.

CONCLUSIONS

We have developed a sensitive and accurate technique for quantitatively determining enprostil diastereomer ratios in soft elastic gelatin capsules. The method provides accurate and precise assays at enprostil concentrations as low as 20% of dosage form labeled strength (= 0.3 mM enprostil). Although resolution between the enprostil isomers depends strongly on system operating conditions, our study of mobile phase composition, temperature, and flow rate effects on analyte retention defines operating ranges under which the method performs satisfactorily. Direct determinations of diastereomer ratios in three lots of enprostil soft elastic gelatin capsules demonstrate that the isomer ratio is constant between different manufacturing lots and that the isomer ratio is invariant with time under various storage conditions.

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