CHROM. 19 200

MACROMOLECULAR PRODRUGS

V. SIMULTANEOUS DETERMINATION OF HIGH-MOLECULAR-WEIGHT DEXTRAN METRONIDAZOLE MONOSUCCINATE ESTER PRODRUGS AND THE HYDROLYSIS PRODUCTS METRONIDAZOLE AND THE CORRESPONDING MONOSUCCINATE ESTER ON NUCLEOSIL DIOL

CLAUS LARSEN* and MARIANNE JOHANSEN

Royal Danish School of Pharmacy, Department of Pharmaceutics, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

(First received September 12th, 1986; revised manuscript received October 16th, 1986)

SUMMARY

A high-performance size-exclusion chromatography procedure using Nucleosil Diol has been developed which provides the simultaneous determination of macromolecular dextran metronidazole monosuccinate ester prodrugs and the hydrolysis products metronidazole and metronidazole monosuccinate. Various factors influencing the chromatographic behaviour of the compounds are discussed. Baseline separation of the three substances was achieved within 8 min by using a 0.05 M phosphate buffer pH 7.5 eluent at a flow-rate of 2 ml min^{-1}. The detection limit at 320 nm for a conjugate with a degree of substitution of 4.61 was found to be 3.5 μg ml^{-1}.

INTRODUCTION

The macromolecular prodrug approach has received growing interest in recent years[1-3]. Among the enormous number of macromolecules tested, dextrans may serve as one of the most promising carrier candidates due to excellent physicochemical properties and pharmacological acceptance[2,4-6]. Although many synthesized conjugates have been evaluated both *in vitro* and *in vivo*, relatively little information is available about the release kinetics of drug compounds from such polymeric carrier systems. In few cases the kinetics of the formation of the active agents from the conjugates have been studied[7-10]. Stability experiments based on direct measurement of the concentration of the intact conjugate have not been reported to our knowledge.

The development of new column packings for high-performance size-exclusion chromatography (HPSEC) has made the separation of various macromolecular compounds possible[11,12]. This development gave inspiration to generate an HPSEC procedure on Nucleosil Diol allowing the simultaneous determination of high-molecular-weight dextran metronidazole monosuccinate ester prodrugs and the hydrolysis products metronidazole and the corresponding monosuccinate ester.

EXPERIMENTAL

Chemicals

Sephadex G-10 and the dextran fractions T-10, T-20, T-40, T-70 and T-500 were purchased from Pharmacia (Uppsala, Sweden). Metronidazole was obtained from Dumex (Copenhagen, Denmark). The synthesis of metronidazole monosuccinate and the corresponding dextran ester conjugates was carried out essentially as previously described[13]. The degree of substitution has been expressed as mg metronidazole monosuccinate released per mg of the conjugate. All other chemicals and buffer substances were of analytical-reagent grade.
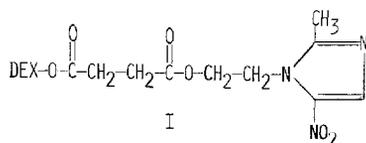
Apparatus and methods

The chromatographic system was composed of a Waters Assoc. Model 6000 A constant-flow pump, a Pye Unicam PU 4020 variable-wavelength detector or a Pye Unicam PU 4023 refractive index detector, and a Rheodyne Model 7125 injection valve. The column, 250 × 8 mm, was packed with spherically shaped Nucleosil Diol 7-OH particles (7 μm) (Macherey-Nagel, Düren, F.R.G.) according to the slurry technique. During chromatography the column was protected by a small pre-column packed with Nucleosil Diol and by a silica saturation column situated between the pump and the injection valve. The latter column was packed with LiChroprep SI 60, 15–25 μm (Merck, Darmstadt, F.R.G.). Optical-rotation and UV-spectral measurements were performed by using a Perkin Elmer Model 141 polarimeter and a Shimadzu UV-190 recording spectrophotometer, respectively. Readings of pH were done on a Radiometer Type pHM 26 instrument.

The degradation experiment was done at 37°C by adding a Dextran T-70 metronidazole monosuccinate ester conjugate (DS 4.61) to a preheated 0.05 M borate buffer solution (pH 8.80 and μ = 0.5) resulting in an initial concentration of the conjugate of approximately 3 mg ml^{-1}. At suitable intervals aliquots were withdrawn and applied to the column. The pseudo-first-order rate constant was calculated from the slope of the logarithm of the peak height of intact dextran derivative *versus* time plot using linear regression.

In all cases the presented results are the average of at least two determinations.

RESULTS AND DISCUSSION

On silica based size exclusion chromatographic packings, such as Nucleosil Diol, LiChrosorb Diol, SynChropak and the TSK-SW series, the highly polar silanol groups have been replaced by less polar, but still hydrophilic, groups in order to minimize molecular interactions of the solute with the active silica surface. The supports have been successfully employed in size separation of proteins and water-soluble ionic polymers[14–17]. Optimal resolution of sample mixtures containing both high and low molecular weight compounds, however, might be complicated as the column packing material, besides exerting a molecular sieve effect, possesses reversed-phase properties[16]. Furthermore, residual silanol groups on the surface of the diol modified silica may lead to anomalous retention behaviour causing either adsorption or repulsion, dependent on the charge of the solutes[14]. The pK_a value of the silanol groups is close to 7 (ref. 14).

$$DEX-O-\overset{O}{\underset{\|}{C}}-CH_2-CH_2-\overset{O}{\underset{\|}{C}}-O-CH_2-CH_2-N\diagup\begin{smallmatrix}CH_3\\N\end{smallmatrix}$$

I   NO$_2$

Parameters affecting the elution profiles of the compounds

The degradation of the synthesized macromolecular prodrugs of metronidazole, *e.g.* dextran metronidazole monosuccinate ester conjugates (Dex-MMS) (I) in aqueous buffer solutions is accompanied by the parallel formation of metronidazole monosuccinate (MMS) (M_w = 271; pK_a^I = 2.6[18], and pK_a^{II} = 4.56[19]) and metronidazole (M_w = 171; pK_a = 2.6[18]). In order to determine the concentration *vs.* time profiles of the individual compounds after incubation of the conjugates in aqueous solutions over a broad pH range by HPSEC, sample solutions of various composition were to be injected into the chromatograph. Using an acidic mobile phase (pH 2.0) the development of unsymmetrical shoulder-like peaks for both low-molecular-weight agents is observed when applied in phosphate and borate buffer (Table I). This phenomenon might be attributed to the partly protonized metronidazole imidazole ring at pH 2. Suppression of the proposed ionization effect is obtained by changing the eluent pH to 7.5. In addition, the pH change causes a reversion of the elution order of metronidazole and MMS implying that the Nucleosil Diol support indeed exhibits reversed-phase properties. The mobile phase pH shift to pH 7.5 does also result in an increment of the retention volume of MMS, although the carboxylic acid group of the latter derivative is fully ionized in weakly alkaline solution. The mechanism responsible for this unexpected chromatographic behaviour is not obvious. In contrast to metronidazole and the monosuccinate ester derivative the elution

TABLE I

EFFECT OF THE COMPOSITION OF THE SAMPLE SOLUTION AND pH OF THE MOBILE PHASE ON THE ELUTION BEHAVIOUR OF METRONIDAZOLE, METRONIDAZOLE MONOSUCCINATE (MMS) AND A DEXTRAN T-70 METRONIDAZOLE MONOSUCCINATE ESTER CONJUGATE (Dex-MMS) WITH DS 4.61

Eluent a: 0.05 M phosphate pH 2.0–2-methoxyethanol (95:5, v/v). Eluent b: 0.05 M phosphate pH 7.5–2-methoxyethanol (95:5, v/v).

Sample solution composition	Metronidazole		MMS		Dex-MMS	
	V_R (ml)	$N^{\star\star}$	V_R (ml)	$N^{\star\star}$	V_R (ml)	$N^{\star\star}$
Eluent[a]	10.4	3530	11.4	5770	5.8	445
Eluent[b]	10.4	3530	11.4	5770	5.8	445
0.05 M Phosphate pH 6.91	10.4[*a]	—	11.5[*a]	—	5.8[a]	445
0.05 M Borate pH 9.14	10.4[*a]	—	11.5[*a]	—	5.8[a]	445
0.05 M Phosphate pH 6.91	13.7[b]	4690	13.2[b]	5690	5.8[b]	295
0.05 M Borate pH 9.14	13.7[b]	4690	13.2[b]	5690	5.8[b]	295

* Unsymmetrical, shoulder-like peaks.
** Number of theoretical plates per 25 cm.

characteristics of the dextran conjugate appear to be relatively insensitive to the variations in the operating conditions shown in Table I.

Because of the contribution of a reversed-phase partition mechanism in chromatography on Nucleosil Diol, the resolution of metronidazole and the corresponding monosuccinate ester can be optimized by variation of the content of the organic component in the mobile phase. In Table II the influence of the 2-methoxyethanol concentration in the eluent on the retention volumes of the various compounds is shown. Although no unambiguous trend in the elution profiles of the three compounds is apparent, increasing the concentration of 2-methoxyethanol in the mobile phase up to 5% results in an enhanced column efficiency as expressed in the number of theoretical plates. The increase in N for the individual substances, however, is obtained at the expense of the resolution of metronidazole and MMS. Significant peak broadening is observed when the content of the organic component in the eluent is raised to 10%. The organic component is adsorbed to the support[20], rendering the diol phase less hydrophilic. Because of the polar nature of the compounds under investigation, altered surface coating properties might be responsible for the diminished chromatographic capacity. Interestingly, the introduction of 2-methoxyethanol in the mobile phase up to 5% produces sharper peaks for all the compounds, but in the case of the conjugate the retention volume is almost unaffected. This observation is in contrast with the chromatography of lysozyme and chymotrypsinogen on LiChrosorb Diol[15]. The enzymes were eluted with water–ethylene glycol (80:20). Incorporation of ethylene glycol in the mobile phase afforded suppression of hydrophobic adsorption effects resulting in a marked decrease of the retention volumes of the proteins. As seen in Table II the use of a mobile phase with a higher ionic strength affected the elution characteristics only to a minor extent, suggesting that ionic interactions between the solutes and the stationary phase are un-likely to contribute to the separation mechanism. The 0.05 M phosphate buffer pH 7.5 eluent which allowed baseline separation of the three compounds (Fig. 1) was selected for further investigations.

TABLE II

INFLUENCE OF THE 2-METHOXYETHANOL CONCENTRATION AND THE IONIC STRENGTH, μ, OF THE 0.05 M PHOSPHATE pH 7.5 BUFFER ELUENT ON THE RETENTION VOLUMES OF METRONIDAZOLE, METRONIDAZOLE MONOSUCCINATE (MMS) AND A DEXTRAN T-70 METRONIDAZOLE MONOSUCCINATE ESTER CONJUGATE (Dex-MMS) WITH DS 4.61

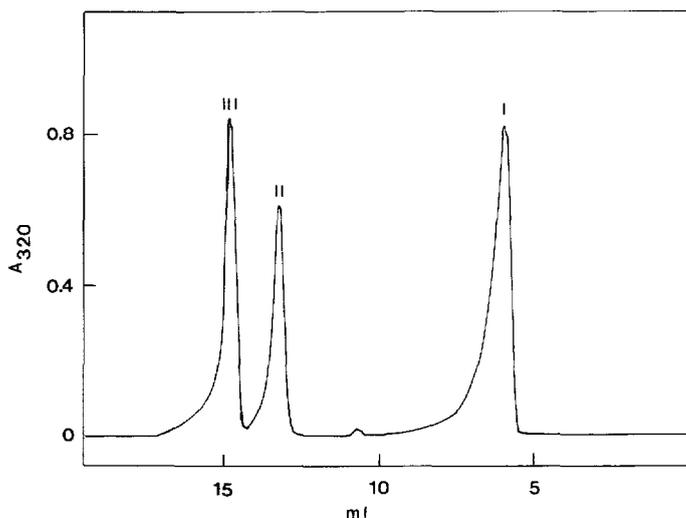| 2-Methoxyethanol content or μ of the mobile phase	Metronidazole		MMS		Dex-MMS	
	V_R (ml)	$N^\star$	V_R (ml)	$N^\star$	V_R (ml)	$N^\star$
10% (v/v)	12.7	4030	11.7	3890	6.4	150
5% (v/v)	13.7	4690	13.2	5690	5.8	445
2.5% (v/v)	14.2	3980	13.4	4690	5.9	285
0%	15.0	3600	13.7	4490	5.9	247
0.3 M sodium chloride	14.6	4010	13.4	4490	6.0	225

$\star$ Number of theoretical plates per 25 cm.

Fig. 1. Separation of an aqueous mixture of Dex T-70-MMS (I) (DS 4.61), MMS (II) and metronidazole (III) on Nucleosil Diol. Eluent: 0.05 M phosphate pH 7.5. Flow-rate: 1 ml min^{-1}.

The influence of the flow-rate on the column efficiency for the individual substances is summarized in Table III. It appears that the chromatographic performance on this modified silica is only moderately affected by variations in the mobile phase velocity from 0.6 to 2.0 ml min^{-1}. In general, flow-rates have more influence on the higher-molecular-weight molecules due to smaller diffusion coefficients. However, the fact that the dextran conjugate ($M_w = 81\ 100$) is at least partly excluded from the stationary phase may account for the nearly constant elution profiles of the conjugate. At a flow-rate of 2.0 ml min^{-1} a separation with sufficient resolution of the compounds is achieved within 8 min.

TABLE III

INFLUENCE OF THE FLOW-RATE ON THE COLUMN EFFICIENCY OF METRONIDAZOLE, METRONIDAZOLE MONOSUCCINATE (MMS) AND A DEXTRAN T-70 METRONIDAZOLE MONOSUCCINATE ESTER CONJUGATE WITH DS 4.61

N is the number of theoretical plates.

Flow-rate (ml min^{-1})	$N_x/N_{1.0}$		
	Dex-MMS	MMS	Metronidazole
0.6	1.07	1.01	1.02
0.8	0.95	1.00	1.04
1.0	1.00	1.00	1.00
1.2	0.95	0.93	0.85
1.4	0.95	0.80	0.80
1.6	0.95	0.78	0.81
1.8	0.94	0.63	0.73
2.0	0.87	0.73	0.77

Quantitation of the derivatives

Linear standard calibration curves obtained by plotting the peak heights *versus* the concentration of the individual compounds are depicted in Fig. 2.
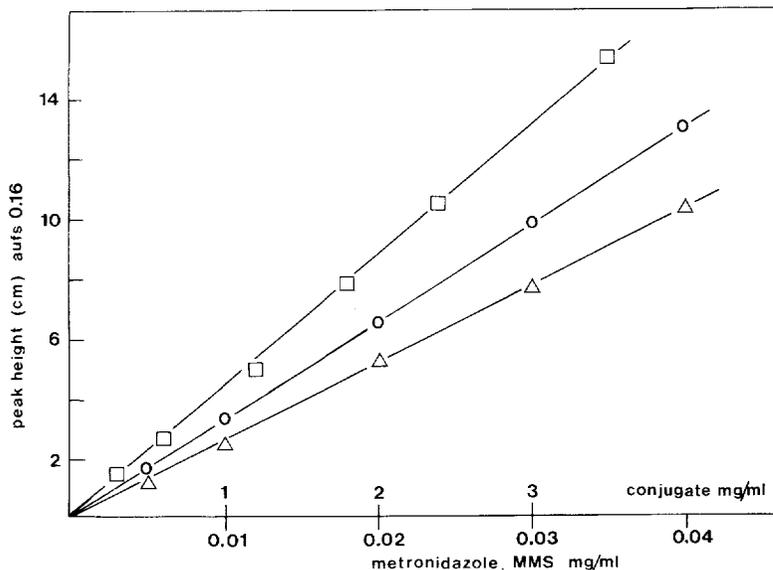

Fig. 2. Standard calibration curves for Dex T-70-MMS (DS 4.61) (□), metronidazole (○) and MMS (△). Aliquots of 20 μl of 0.05 M phosphate buffer pH 6.8 samples were injected. Detection: A_{320}. Eluent: 0.05 M phosphate pH 7.5. Flow-rate 1 ml min^{-1}.

A satisfactory calibration curve is obtained for the dextran T-70 conjugates, which elute close to the exclusion volume of the column. For conjugates synthesized from lower-molecular-weight dextran fractions, however, it is recommended to base quantitative determinations on peak area measurements. This is evident from Fig. 3 which shows the appearance of relatively broad chromatographic peaks for the conjugates derived from the dextran fractions T-10 to T-40. Additionally, in the hydrolysis experiment of the dextran T-70 metronidazole monosuccinate ester derivative (discussed below) a slight deviation from linearity was observed when the quantitation was founded on peak-height measurements.

The detection limits of the conjugates in aqueous buffer solutions depend on the degree of substitution and on the polydispersity of the parent dextran. Using a 100 μl loop 3.5 μg ml^{-1} of a Dex-70-MMS derivative (DS = 4.61; M_w/M_n = 1.93) was detectable.

Log M_w — V_R calibration curves

Separate rectilinear relationship between log M_w and the retention volumes of the Dex-MMS conjugates and the parent dextran T-fractions were observed (Fig. 4). The linear calibration curves can be described by the following equations:

$$\log M_w \text{ (conjugate)} = 6.80 - 0.31 \ V_R \ (n = 4, r = 0.997) \tag{1}$$

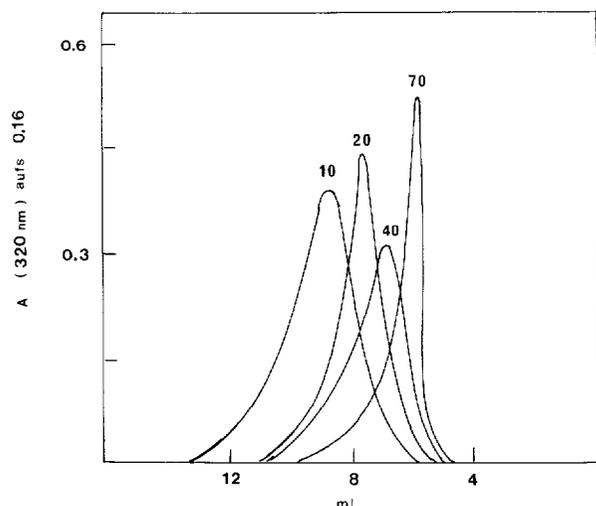$$\log M_w \text{ (dextran)} = 7.26 - 0.40 \ V_R \ (n = 4, r = 0.999) \tag{2}$$

Fig. 3. Elution profiles for Dex-MMS conjugates with varying molecular weights. Based on the parent dextran properties; T-70 (DS 8.43, M_w = 75 200, M_w/M_n = 1.93); T-40 (DS = 11.1; M_w = 43 900, M_w/M_n = 1.68); T-20 (DS = 7.5, M_w = 20 400, M_w/M_n = 1.24); T-10 (DS = 7.7, M_w = 10 300, M_w/M_n = 2.10). Eluent: 0.05 M phosphate pH 7.5. Samples (20 μl) containing approximately 5 mg ml^{-1} were injected.

The void volume, V_0, was found to be 5.85 ml, corresponding to the retention volume of a dextran T-500 (M_w = 500 000) sample. The retarded elution of the Dex-MMS conjugates in proportion to the unsubstituted dextrans is most likely due to the reversed-phase properties of the column packing material. In addition, introduction of substituents into the dextran matrix may alter the original random coil structure due to intramolecular interactions[11]. A resulting reduction of the hydrodynamic volumes of the conjugates may therefore also contribute to the observed enlargement of the
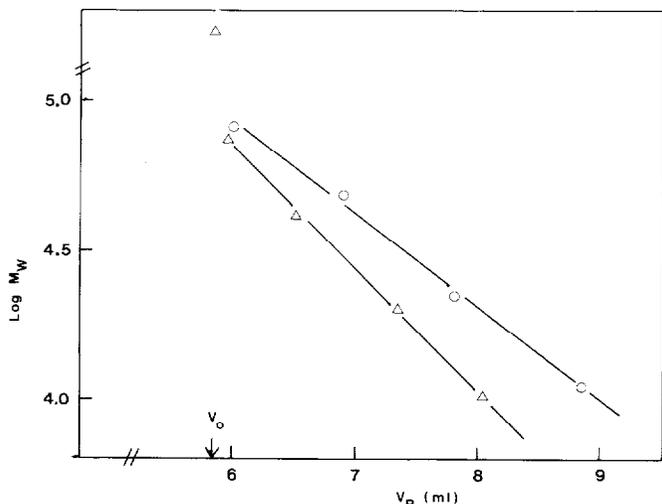

Fig. 4. Molecular weight calibration curves for Dex-MMS conjugates (○) and the parent dextrans (△). V_0 determined by V_R for dextran T-500 (M_w = 500 000). See Fig. 3 for data of the other compounds.

retention volumes. The latter suggestion is supported by earlier studies[21], which revealed that in the case of benzoyl dextran ester derivatives the hydrodynamic volume, expressed by the limiting viscosity number, is almost inversely proportional to the degree of substitution. The discrepancy between the slopes of the two calibration curves cannot easily be explained, but it may support the observation that non-size-exclusion effects contribute to the complex separation mechanism.

Kinetics of hydrolysis

The kinetics of hydrolysis of a Dex-MMS conjugate (DS = 4.61) was studied in 0.05 M borate buffer pH 8.80 ($\mu = 0.5$ and 37°C). The degradation reaction displayed first-order kinetics over approximately two half-lives as evidenced by the linear plot of log p_t against t (Fig. 5). The pseudo-first-order rate constant, k_{obs}, was calculated to be 0.48 h^{-1}. Using the

$$\log a_{OH} = pH - 13.62 \tag{3}$$

relationship[22] where a_{OH} refers to the hydroxide ion activity, a value of $32.4 \cdot 10^4$ M h^{-1} for the second order rate constant for specific base catalyzed hydrolysis, k_{OH}, was found. In comparison, the latter rate constant is observed to be of the same order of magnitude as the k_{OH}-value reported earlier for hydrolysis of Dex-MMS[10] (determined by the initial rate method).

In Fig. 5 peak heights (p_t) have been taken as a measure of the concentration of intact dextran conjugate at time t. A slight deviation from linearity is observed for the data derived from the early part of the degradation experiment. A better linear correlation is obtained by replacing peak heights by peak areas (using the "cut and weight" method). This finding indicates that a small change of the elution behaviour of the conjugate occurs during the early stages of the degradation. The chem-
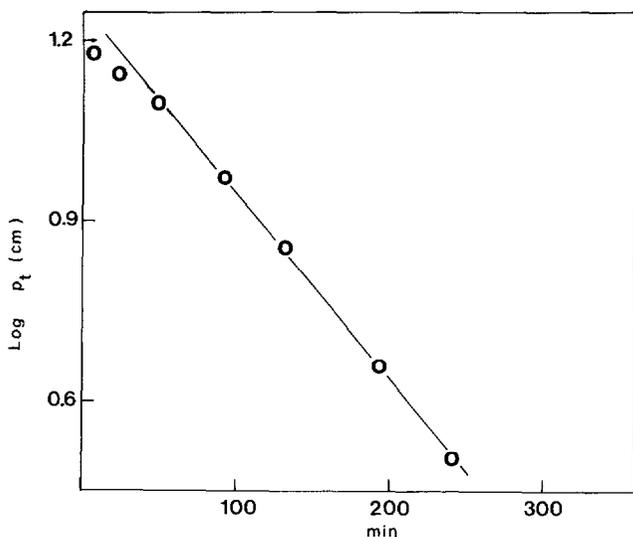

Fig. 5. First-order plot for hydrolysis of a Dex 70-MMS conjugate (DS 4.61) in 0.05 M borate buffer pH 8.80 (37°C and $\mu = 0.5$). p_t corresponds to the peak height of intact conjugate at time t.

ical structure of Dex-MMS changes continuously during hydrolysis. The initial variation of the elution profiles might therefore be a reflection of the alteration of the adsorptive and hydrodynamic properties of the derivatives due to hydrolysis of the ligand and to the intermediate formation of ionized carboxylic acid groups.

ACKNOWLEDGEMENT

REFERENCES

1 M. J. Poznansky and L. G. Cleland, in R. L. Juliano (Editor), *Drug Delivery Systems,* Oxford University Press, New York, 1980, p. 253.
2 H. Sezaki and M. Hashida, *CRC Critical Reviews in Therapeutic Drug Carrier Systems,* 1 (1984) 1.
3 M. J. Poznansky and R. L. Juliano, *Pharmacol. Rev.,* 36 (1984) 277.
4 A. D. Virnik, K. P. Khomyakov and I. F. Skokova, *Russ. Chem. Rev.,* 7 (1975) 1280.
5 L. Molteni, in G. Gregoriadis (Editor), *Drug Carriers in Biology and Medicine,* Academic Press, London, 1979, p. 107.
6 C. Larsen and M. Johansen, *Arch. Pharm. Chemi,* 92 (1985) 809.
7 M. Hashida, Y. Takakura, S. Matsumoto, H. Sasaki, A. Kato, T. Kojima, S. Muranishi and H. Sezaki, *Chem. Pharm. Bull.,* 31 (1983) 2055.
8 C. Larsen and M. Johansen, *Int. J. Pharm.,* 27 (1985) 205.
9 C. Larsen, M. Johansen and E. Harboe, *Arch. Pharm. Chemi. Sci. Edn.,* 14 (1986) 44.
10 C. Larsen and M. Johansen, *Int. J. Pharm.,* (1987) in press.
11 H. G. Barth, *J. Chromatogr. Sci.,* 18 (1980) 409.
12 J. Lesec, *J. Liq. Chromatogr.,* 8 (1985) 875.
13 J. Vermeersch, F. Vandoorne, D. Permentier and E. Schacht, *Bull. Soc. Chim. Belg.,* 94 (1985) 591.
14 P. Roumeliotis and K. K. Unger, *J. Chromatogr.,* 185 (1979) 445.
15 D. E. Schmidt, Jr., R. W. Giese, D. Conron and B. L. Karger, *Anal. Chem.,* 52 (1980) 177.
16 D. P. Herman and L. R. Field, *J. Chromatogr. Sci.,* 19 (1981) 470.
17 R. P. Frigon, J. K. Leypoldt, S. Uyejl and L. W. Henderson, *Anal. Chem.,* 55 (1983) 1349.
18 M. J. Cho, R. R. Kurtz, C. Lewis, S. M. Machkovech and D. J. Houser, *J. Pharm. Sci.,* 71 (1982) 410.
19 M. Johansen and C. Larsen, *Int. J. Pharm.,* 21 (1984) 201.
20 W. Mönch and W. Dehnen, *J. Chromatogr.,* 147 (1978) 415.
21 M. Johansen and C. Larsen, *Int. J. Pharm.,* 27 (1985) 219.
22 H. S. Harned and W. J. Hamer, *J. Am. Chem. Soc.,* 55 (1933) 2194.