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HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF FLUORESCEINYL ISOTHIOCYANATE DEXTRANS OF VARIOUS MOLECULAR MASSES IN BIOLOGICAL MEDIA

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SUMMARY

A high-performance size-exclusion chromatographic procedure, using Nucleosil Diol, for the quantitative analysis of fluoresceinyl isothiocyanate dextrans of various molecular masses (10 000–150 000) in biological media was developed. The influence of the molecular mass and the degree of substitution of the conjugates on the chromatographic behaviour are discussed. In addition to quantitation, the molecular mass of the conjugates with degree of substitution below 1.6 could be estimated from the chromatograms. Linear standard calibration curves were obtained at concentrations down to 0.050 $\mu g\ ml^{-1}$ in rabbit plasma and urine and homogenates of rabbit liver, lymph node and muscle when the derivative (degree of substitution 0.85) was monitored by fluorescence detection ($\lambda_{ex} = 493$ nm, $\lambda_{em} = 520$ nm). The fluoresceinyl isothiocyanate dextrans were found to be stable for more than three days at 37°C in all biological media under investigation. A pH–rate profile for the alkaline hydrolysis of fluoresceinyl isothiocyanate dextrans was constructed. The applicability of the method to pharmacokinetic studies was demonstrated by recording the plasma concentration–time profile of a fluoresceinyl isothiocyanate dextran T-70 conjugate following intravenous injection to a rabbit. In relation to future pharmacokinetic investigations on dextran conjugates, the results reported indicate that labelling of the parent dextran with fluoresceinyl isothiocyanate and monitoring of the fluoresceinyl isothiocyanate dextran conjugate throughout the organism using the described method is a promising development

INTRODUCTION

Although macromolecular prodrugs with dextran as the carrier group have been extensively evaluated in recent years [1–3], few studies have dealt with the in vivo disposition of the conjugates following parenteral administration.

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In order to perform such investigations, a method for analysis of the dextran derivatives in biological media is needed. One possibility would be to monitor the conjugates according to dextran per se. Dextran is, however, quantitated by a rather tedious and unspecific procedure [4]. As an alternative principle, it has become attractive to label the parent dextran with a tracer. Dextran conjugates in which mitomycin C was attached to [^{14}C]dextran [5] and [^{125}I]carboxypeptidase G_2 attached to [^{14}C]dextran [6] have been employed in pharmacokinetic studies. However, the analytical procedures were unable to discriminate between the intact conjugate and fragments that resulted from enzymic breakdown of the compounds. Recently we have successfully generated a high-performance size-exclusion chromatographic (HPSEC) procedure on Nucleosil Diol for the determination of dextran–naproxen ester prodrugs [7], the compounds being monitored by fluorescence detection. These conjugates, however, were not sufficiently stable to act as tracers in vivo. The HPSEC principle was also used by Worrell and co-workers [8,9] in two investigations on the pharmacokinetics of antibody–ricin A chain conjugates. The latter workers employed a TSK G3000 SW column and measured the radioactivity of collected fractions.

Fluoresceinyl isothiocyanate (FITC) dextrans were used as tracer substances in investigations of the nervous system [10,11], in microcirculatory research [12–14], in studies dealing with the permeability of various endogenous membranes [15–17], and in the elucidation of the uptake mechanism of macromolecules by different types of cells [18,19]. Although the molecule is negatively charged at physiological conditions, it seems probable that FITC-dextrans behave similarly to the parent compound in vivo [20]. Thus, this conjugate might fulfil the requirements of a dextran tracer that, following further derivatization, could be determined according to the FITC-dextran moiety.

In previous reports, the compound was quantitated by fluorescence spectrophotometry [15,20,21]. However, this principle has at least two disadvantages. First, the method does not give any information on the molecular mass of the conjugate and consequently a possible breakdown of the dextran backbone would not be detected. Second, a spectrophotometric method might not be applicable for a specific determination of the conjugate in an extensive range of biological media, owing to the possibility of interference from other fluorescent species in the media. Therefore, we decided to apply the HPSEC principle to design a method to detect FITC-dextran in a reproducible, specific and sensitive manner in biological media.

EXPERIMENTAL

Chemicals

FITC was obtained from Sigma (St. Louis, MO, U.S.A.). Sephadex G-10 and dextran fractions with average molecular masses (M_r) of ca. 10 000 (T-

10), 20 000 (T-20), 40 000 (T-40) and 70 000 (T-70) were obtained from Pharmacia (Uppsala, Sweden). FITC-dextrans were either purchased from Sigma or synthesized essentially as previously described [21] and purified by gel filtration on Sephadex G-10 prior to lyophilization. The degree of substitution (DS) was determined as proposed by De Belder and Granath [21] and was expressed as the amount (in milligrams) of the FITC ligand per milligram of conjugate, in percentage terms. All other chemicals used were of analytical grade.

Equipment and chromatographic system

The Hitachi chromatographic system was composed of a Model 655 A-11 liquid chromatograph, a Model F-1000 fluorescence detector, a Model D-2000 chromato-integrator, and a Rheodyne Model 7125 injection valve with a 20-μl loop. The column (250 mm$\times$8 mm I.D.) was slurry-packed with spherical Nucleosil Diol 7-OH particles (7 μm) (Macherey-Nagel, Düren, F.R.G.). During chromatography the column was protected by a small guard 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.). The mobile phase consisted of a 0.02 *M* phosphate buffer (pH 6.5), and the flow-rate was 1.0 ml min^{-1}. The column effluent was monitored at excitation and emission wavelengths of 493 and 520 nm, respectively. Spectrophotometric measurements were done on a Shimadzu UV-190 recording spectrophotometer. Spectrofluorometric measurements were carried out by using a Perkin-Elmer 1000 fluorescence spectrophotometer. A Radiometer Type pH M 62 meter was used to record the pH at the temperature of study.

Sample preparation

A 500-μl volume of all biological samples was deproteinized by 200 μl of 20% trichloroacetic acid, vortex-mixed and centrifuged (3 min, 10 000 *g*), and then 20 μl of the supernatant were injected into the chromatograph.

Kinetics of degradation of FITC-dextran

Kinetic measurements were performed under the following conditions: carbonate buffers and sodium hydroxide solutions were used for maintaining the pH, the reaction solutions being kept at a constant temperature of 37°C. The reactions were initiated by adding the appropriate FITC-dextran to the preheated buffer to yield an initial concentration of the conjugate of ca. 5 μg ml^{-1}. At suitable intervals, aliquots were withdrawn and injected into the chromatograph. Pseudo-first-order rate constants were calculated from linear plots of ln A_t versus time, where A_t is the peak area of the remaining intact dextran derivative at time t.

Degradation experiments were done at 37°C and pH 7.4 in 80% rabbit plasma

(adjusted to pH 7.4 by addition of an appropriate amount of 0.2 *M* phosphate buffer, pH 7.40), 10% rabbit muscle homogenate, 10% rabbit lymph node homogenate, 10% rabbit liver homogenate and in rabbit urine (pH 8.0). The tissue homogenates were prepared by suspending each organ in twice the volume of cold 0.9% sodium chloride. The mixture was homogenized and centrifuged at 5000 *g* in a refrigerated (4°C) centrifuge. The resulting supernatant was frozen immediately in 2-ml portions. Immediately before the experiments the latter homogenates were diluted with 0.2 *M* phosphate buffer (pH 7.40) to give the respective 10% tissue homogenates. After incubation of the derivatives (100 μg ml^{-1}), 500-μl samples were withdrawn at suitable intervals and handled as described above.

In vivo experiment

One male rabbit weighing 2.5 kg was used for the experiment: 1.0 ml of a 10% (w/v) solution of FITC-dextran T-70 (DS 2.1) in 0.9% sodium chloride was injected into the auricular vein. Blood samples were taken by vein puncture from the corresponding vein in the opposite ear. The samples were centrifuged in heparinized test-tubes (4 min, 10 000 *g*), and 500 μl of plasma were analysed as described above.

RESULTS AND DISCUSSION

Mobile phase composition

FITC-dextran (Fig. 1) can be monitored by fluorescence detection (λ_{ex}=493 nm, λ_{em}=520 nm). The ionization state of the carboxylate group in the mol-

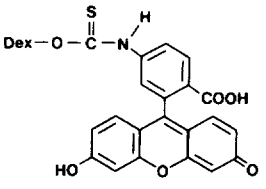

Fig. 1. Structure of FITC-dextran.

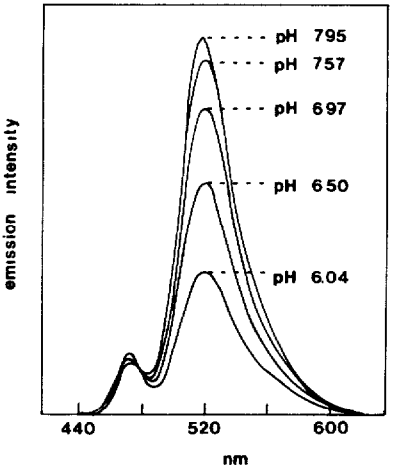

Fig. 2. pH dependence of the fluorescence emission spectrum of a FITC-dextran T-40 conjugate (DS 0.7); λ_{ex}=471 nm.

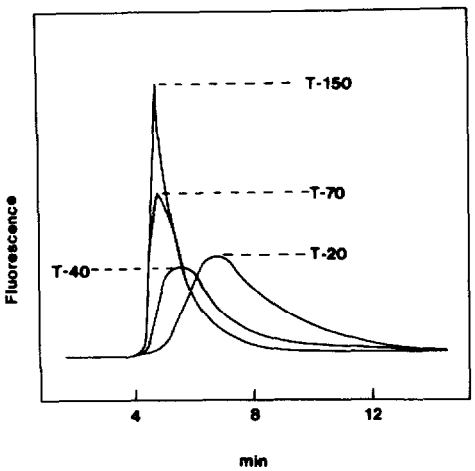

Fig. 3. Elution profiles for FITC-dextrans of various molecular masses: T-150 (DS 1.0), T-70 (DS 0.85), T-40 (DS 0.7) and T-20 (DS 1.1).

ecule affects the delocalization of the π-electrons in the polycyclic aromatic system. Thus, as shown in Fig. 2, the fluorescence emission of the FITC-dextran strongly depends on the pH. In order to obtain a maximum sensitivity, a pH of 8.0 should be selected for the mobile phase. However, we observed that the life-span of the column was increased by maintaining the pH of the eluent somewhat below the neutral point. As a compromise, a 0.02 *M* phosphate buffer of pH 6.5 was chosen as the mobile phase. The chromatograms obtained showed a single peak corresponding to the individual FITC-dextran derivative. The retention times varied between 4.5 and 9.0 min, depending on the M_r of the individual conjugates (DS < 1.6). Typical chromatograms of conjugates with different molecular masses are presented in Fig. 3.

Effect of molecular mass and degree of substitution of the conjugates

The effect of M_r and DS of the conjugates on the detector response were evaluated by recording the peak areas corresponding to equal amounts (6 μg ml^{-1}) of FITC-dextrans T-10 (DS 1.6), T-20 (DS 1.1), T-40 (DS 0.7 and 2.1), T-70 (DS 0.85, 2.1 and 3.2) and T-150 (DS 1.0) (Fig. 4). For DS below ca. 1.6, the peak areas were independent of M_r (10 000–150 000) of the carrier molecule, but strictly proportional to DS of the conjugates. In the case of more substituted dextran derivatives deviation from linearity is observed, indicating that elution of the latter compounds is not governed exclusively by a size-exclusion mechanism. In a previous study [7], linear plots of peak area versus DS were obtained for dextran–naproxen ester prodrugs (DS up to 10) by employing an aqueous mobile phase containing 30% (v/v) acetonitrile. Since we have focused on the development of an assay for FITC-dextrans of fairly low

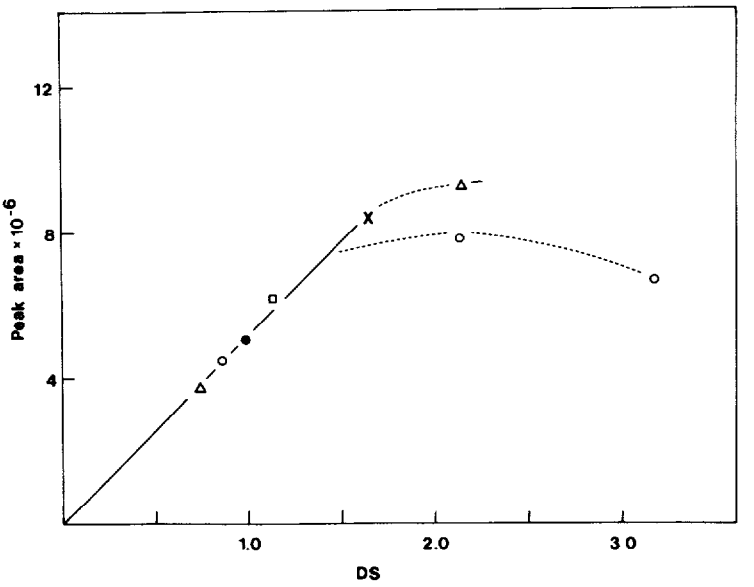

Fig. 4. Dependence of the peak area on the degree of substitution of FITC-dextran. The concentration of all the compounds was 6 μg ml^{-1}: (×) T-10; (□) T-20; (△) T-40; (○) T-70; (●) T-150.

DS, which are assumed to mimic the pharmacokinetic properties of the parent dextran, no attempts were made to include organic solvents in the eluent.

In addition to the molecular size, the retention times of the conjugates, t_R, are also influenced by the DS of the derivatives (Fig. 5). When the DS is kept low and within a narrow range (0.7–1.1), a linear relationship apparently exists between log M_r and t_R ($n=3$, $r=0.99$) in the 10 000–70 000 M_r range. Although further data are necessary to establish an equation for the log M_r–t_R correlation, the present data indicate that information on the molecular masses of the conjugates might be deduced from the chromatograms. Contrary to this, the previously described spectrophotometric method for quantitation of FITC-dextran [20] was unable to discriminate between conjugates of different molecular masses.

A similar plot for a second series of data (conjugates with DS 1.6–2.2) is also included in Fig. 5. It is apparent that for compounds of comparable size a higher DS results in an enhanced retention time. The discrepancy between the slopes of the two lines suggests that variation of DS affects the retention times of the low-molecular-mass derivatives to a greater extent. This influence of DS on the elution behaviour of the FITC-dextrans is most likely due to adsorptive interaction between the FITC ligands and the packing material, which, apart from exerting a molecular sieve effect, also possesses reversed-phase properties [22,23].

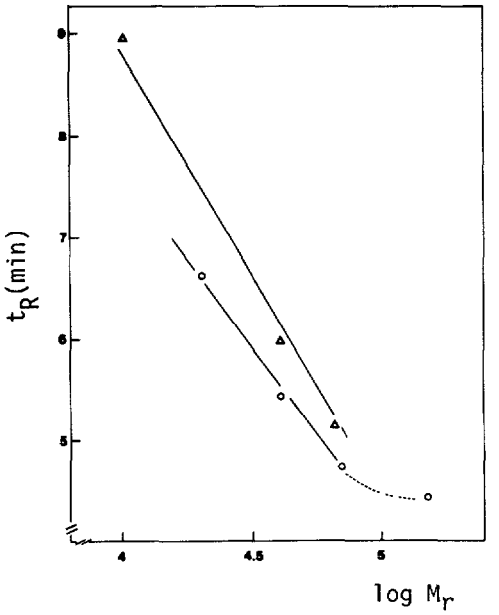

Fig. 5. Dependence of the retention time on the logarithm of the molecular mass of the FITC-dextran conjugates: (○) DS 0.7–1.1; (△) DS 1.6–2.1.

Stability of FITC-dextrans

The stability of FITC-dextrans was investigated in various biological media at 37°C. No significant change was seen in either the retention time or the peak area for at least three days in 80% rabbit plasma, 10% rabbit muscle homogenate, 10% rabbit liver homogenate, 10% rabbit lymph node homogenate and in rabbit urine, indicating that both the thionocarbamoyl linkage and the dextran backbone are stable under these experimental conditions. This is in agreement with previous reports [20,21], which concluded that FITC-dextrans are stable for at least one day at physiological conditions. No difference in stability was seen between conjugates of different M_r or DS. Furthermore, no change of the two parameters was observed when a 10 μg ml^{-1} solution of FITC-dextran T-70 in 6% trichloroacetic acid was allowed to stand at room temperature for three days. Thus, the deproteinization step in the analytical procedure does not disturb the stability of the sample solution.

The kinetics of hydrolysis of FITC-dextran was investigated at 37°C in aqueous buffer (pH 10.01–13.30). The degradation reactions displayed first-order kinetics over the first one or two half-lives. The pH–rate profile for decomposition of FITC-dextran is shown in Fig. 6. A specific catalysis by hydroxide ions (slope = 0.99) evidently occurs in the pH range 10.0–10.75. Assuming that this relationship might be extended to be valid at pH 7.4, a half-

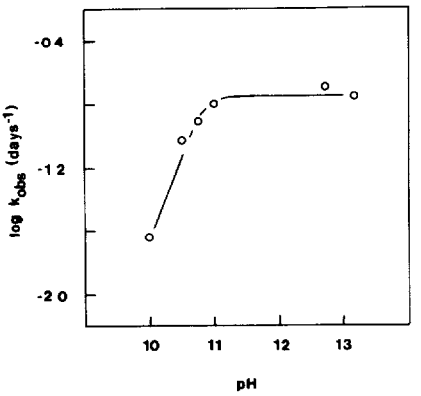

Fig. 6. pH–rate profile for the hydrolysis of a FITC-dextran T-70 conjugate (DS 0.85) at 37 °C.

life of 30 years is calculated for the hydrolysis of the conjugate at physiological pH.

Structurally, FITC-dextran is an N-monosubstituted thionocarbamate, and pH–rate profiles similar to the one presented here were found for the hydrolysis of N-acetyl-carbamates [24] and phenylthionocarbamates [25]. The rate constants of alkaline hydrolysis of N-monosubstituted carbamates derived from aliphatic ($pK_a \approx 16$) and aromatic alcohols ($pK_a \approx 10$) differ by a factor 10^6–10^7 [26]. We have recently determined the pK_a of dextran to be 11.8 (to be published elsewhere). Thus, the finding of a rate of hydrolysis of FITC-dextran between the analogous rates for the ethyl- and phenylcarbamates studied by Dittert and Higuchi [26] seems to be reasonable.

Quantitation of FITC-dextrans

A typical example of a linear standard calibration curve obtained by plotting the peak area or the peak height versus the concentration of the conjugate is shown in Fig. 7. Linearity was found at concentrations down to 0.050 $\mu g\ ml^{-1}$. Although both the peak height and the peak area were proportional to the concentration of the FITC-dextran, quantitation of the compounds was based on peak-area measurements throughout this study. FITC-dextrans were quantitated in aqueous solution, in rabbit plasma and urine and in rabbit liver, muscle and lymph node homogenates. In all investigations we found a linear relationship between the peak area and the concentration of the conjugate. Data are listed in Table I. In certain conditions the resulting chromatograms showed no peaks interfering with the peak corresponding to FITC-dextran. However, some additional peaks were seen in the case of samples originating from liver homogenate and from urine. Retention times of the unknown substances varied from 10 to 35 min. These peaks were probably caused by low-molecular-mass components of the media and could not be removed by raising

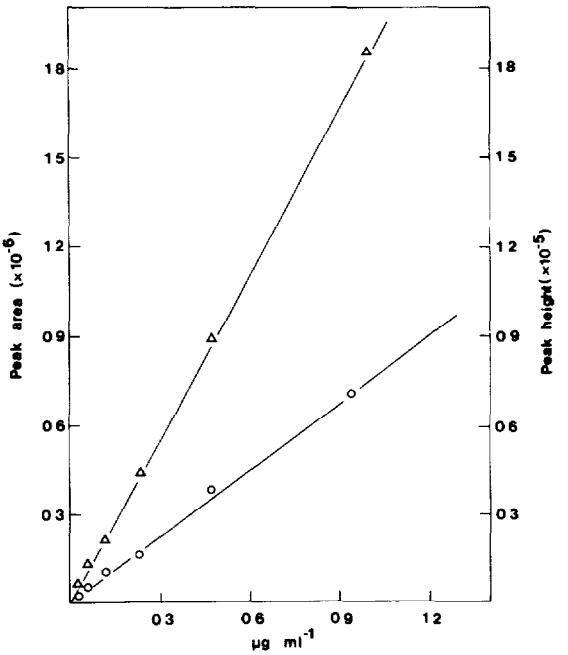

Fig. 7. Standard calibration curves for a FITC-dextran T-70 conjugate (DS 0.85) in rabbit plasma: (○) peak area; (△) peak height.

TABLE I

ANALYTICAL DATA FOR THE QUANTITATION OF A FITC-DEXTRAN T-70 CONJUGATE (DS 0.85) IN WATER AND VARIOUS RABBIT SAMPLE MEDIA

Sample medium	α^a (area $\times 10^{-6}$) (μg^{-1} ml)	Recovery[b] (%)	Detection limit (μg ml^{-1})	Reproducibility (s_{rel}, %)
Water	1.051	100	0.025	0.74[c]/2.1[d]
Plasma	0.760	99	0.050	3.7[e]/7.9[d]
25% Liver homogenate[f]	0.840	109	0.050	—
10% Muscle homogenate[f]	0.768	100	0.050	—
10% Lymph node homogenate[f]	0.697	90	0.050	—
Urine	0.770	100	0.050	—

[a] $n=7$ and $r=0.99$.
[b] Calculated from the slopes of the calibration curves.
[c] 10 μg ml^{-1}, area.
[d] 0.050 μg ml^{-1}, height.
[e] 1 μg ml^{-1}, area.
[f] Adjusted to pH 7.4 by addition of appropriate amounts of 0.2 *M* phosphate buffer (pH 7.40).

five-fold the volume of trichloroacetic acid added to the sample. The presence of these species in urine and in liver homogenate indicates that quantitation of the derivatives in such media by using the previously described spectrofluorometric principle [20] is subject to some uncertainty.

The detection limit depends on the degree of substitution of the conjugates. It is seen from Table I that the assay enables detection of a FITC-dextran T-70 conjugate (DS 0.85) in concentrations of 0.025 and 0.050 $\mu g\ ml^{-1}$ in aqueous solution and in biological media, respectively. The difference between these detection limits is predominantly a consequence of the dilution of the biological samples in the precipitation step. As seen from Table I, the reproducibility of the assay procedure is acceptable, and recoveries are close to 100% in all media.

Applicability of the method in pharmacokinetic studies

The disappearence of FITC-dextran T-70 (DS 2.1) from the circulation following intravenous injection to one rabbit was monitored. The resulting plasma concentration–time profile is shown in Fig. 8. The data reflect a first-order elimination of FITC-dextran from the blood stream with a half-life of 63 min. This method is applicable to further studies on the pharmacokinetics of dextran conjugates.

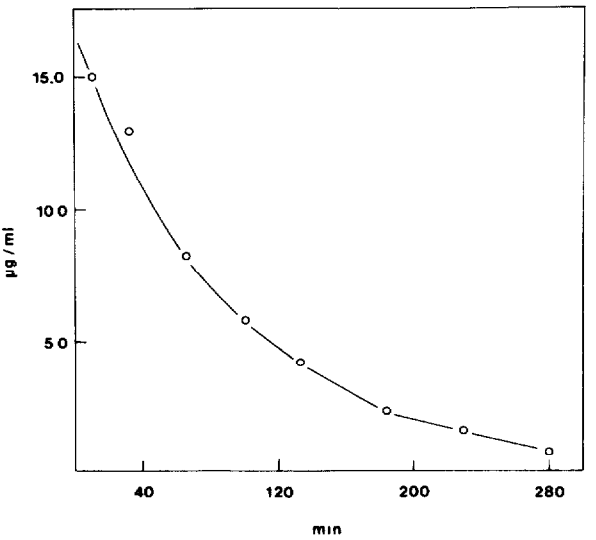

Fig 8. Plasma concentration–time course of a FITC-dextran T-70 conjugate (DS 2.1) following intravenous injection of a 100-mg dose to a male rabbit.

REFERENCES

1 C Larsen and M. Johansen, Arch. Pharm. Chem., 92 (1985) 809.
2 H. Sezaki and M. Hashida, CRC Crit. Rev. Ther. Drug Carrier Systems, 1 (1984) 1.
3 M. Hashida, A. Kato, Y. Takakura and H. Sezaki, Cancer Res., 44 (1984) 492.
4 G. Wallenius, Acta Soc. Med. Ups., 59 (1953) 69.
5 M. Hashida, A. Kato, Y. Takakura and H. Sezaki, Drug Metab. Dispos., 12 (1984) 492.
6 R.G. Melton, C.N Wiblin, A. Baskerville, R.L. Foster and R.F. Sherwood, Biochem. Pharmacol., 36 (1987) 113.
7 C. Larsen, J. Pharm. Biomed. Anal., (1989) in press.
8 N.R. Worrell, A.J. Cumber, G.D. Parnell, A. Mirza, J.A. Forrester and W.C.J. Ross, Anti-Cancer Drug Design, 1 (1986) 179.
9 N.R. Worrell, A.J. Cumber, G.D. Parnell, W.C.J. Ross and J.A. Forrester, Biochem. Pharmacol., 35 (1986) 417.
10 J.C. Glover, G. Petursdottir and J.K.S. Jansen, J. Neurosci. Methods, 18 (1986) 243.
11 D. Hultström, L. Malmgren, D. Gilstring and Y. Olsson, Acta Neuropathol. (Berlin), 59 (1983) 53.
12 Y. Hamada, T. Otori, T. Suzuki and K. Okamoto, J. Hypertens., 4 (1986) 417.
13 S.L. Lightman, L.E. Caspers-Velu, S. Hirose, R.B. Nussenblatt and A.G. Palestine, Arch. Ophthalmol., 105 (1987) 844.
14 K.E. Arfors and H. Hint, Microvasc. Res., 3 (1971) 440.
15 H. Yoshikawa, K. Takada and S. Muranishi, J. Pharm. Dyn., 7 (1984) 1.
16 B. Persky and D.M. Grganto, Clin. Exp. Metastasis, 5 (1987) 321.
17 J.P. Camilleri, M.O. Nlom, D. Joseph, J.B. Michel, D. Barres and J. Mignot, Exp. Mol. Pathol., 39 (1983) 89.
18 R.A. Preston, R.F. Murphy and E.W. Jones, J. Cell. Biol., 105 (1987) 1981.
19 J.A. Oliver, R.D. Berlin and B.H. Davis, Methods Enzymol., 108 (1984) 336.
20 U. Schröder, K.E. Arfors and O. Tangen, Microvasc. Res., 11 (1976) 33.
21 A.N. de Belder and K. Granath, Carbohydr. Res., 30 (1973) 375.
22 C. Larsen and M. Johansen, J. Chromatogr., 389 (1987) 227.
23 D.P. Herman and L.R. Field, J. Chromatogr. Sci., 19 (1981) 470.
24 M. Bergon and J.-P. Calmon, Bull. Soc. Chim. Fr., (1976) 797.
25 G. Sartore, M. Bergon and J.-P. Calmon, Tetrahedron Lett., 36 (1974) 3133.
26 L.W. Dittert and T. Higuchi, J. Pharm. Sci., 52 (1963) 852.