Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 798 (2003) 217–222
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Chromatographic determination of the association constant between 8-methoxypsoralen and modified β-cyclodextrin: protective effect of hydroxypropyl-β-cyclodextrin on 8-methoxypsoralen toxicity in human keratinocytes

Y. Guillaume*, C. Andre, N. Simon, A. Gehin, C. Guyon, M. Thomassin, L. Ismaili, F. Aubin, L. Nicod

Equipe des Sciences Séparatives et Biopharmaceutiques, Faculté de Médecine et de Pharmacie, Place Saint Jacques, 25030 Besançon Cedex, France

Received 18 June 2003; received in revised form 28 August 2003; accepted 22 September 2003

Abstract

The retention of 8-methoxypsoralen (8-MOP) on an immobilised hydroxypropyl-β-cyclodextrin (HP-β-CD) column was analysed in HPLC by the determination of its Langmuir distribution isotherm. A such method was used to confirm the potential drug complexing role of this cyclodextrin. The 8-MOP/HP-β-CD association constant (*K*) was equal to 29.5 and 18.7 M^{-1}, respectively, at a temperature equal to 5 and 25 °C, respectively. These association constant values were used to determine the cytotoxicity profile of human keratinocyte cell line (HaCaT) in relation to the complex concentration. It was showed through these data that HP-β-CD had a cytoprotective since a reverse effect of HP-β-CD on 8-MOP cytotoxicity was observed.

Keywords: Association constant; 8-Methoxypsoralen; Hydroxypropyl-cyclodextrin

1. Introduction

Psoralens are regularly used in therapy, in combination with ultraviolet A (UVA) light irradiation to treat skin diseases, such as psoriasis, vitiligo and mycosis fungoides [1–5]. In the skin, the combination of psoralens and UVA, also known PUVA, is a potent modulator of epidermal cell growth and differentiation [6,7]. One commonly used psoralen derivatives is 8-MOP which has a high toxicity dependent to the UV rays but the precise mechanisms underlying its action are not known [8,9]. More than burning, psoralens can induce a carcinoma [10,11]. The therapeutic effectiveness of treatment with 8-MOP plus UV light in wavelengths ranging from 320 to 400 nm (PUVA) is thought to correlate with the formation of lesions in DNA of the epidermal cells [12,13]. Although a major therapeutic mechanism of 8-MOP/UVA might be its antiproliferative effect based on DNA crosslinking, its side effect is the recurrent toxicity of 8-MOP in patients treated by PUVA therapy. Moreover, the poor aqueous solubility of 8-MOP poses bioavailability problems in vivo. Cyclodextrins (CDs), which are torus-shaped cyclic oligosaccharides consisting of six or more α-1,4-linked D-glucopyrannose units, are one of the well-known host molecules capable of forming an inclusion complex (host–guest complex) with a wide variety of organic molecules or so called guest molecules [14]. Several reports have shown the interest of the use of modified β-cyclodextrin in pharmaceutical formulation to improve the bioavailability of drugs and decrease their toxicity [14–20]. Then, the complexation of 8-MOP with HP-β-CD seems to be interresting to investigate. In the first time, it was usefull to determine the association constant between the modified cyclodextrine and 8-MOP. In the literature, Various methods have been reported to calculate the association constants∗ between cyclodextrin and drugs. UV-Vis absorption, NMR, potentiometry fluorescence mea-

* Corresponding author. Tel.: +33-381665546; fax: +33-381665655.
E-mail address: yves.guillaume@univ-fcomte.fr (Y. Guillaume).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.09.045

surements, capillary electrophoresis and calorimetry have been described [12–21]. Usually, for the chromatographic experiments, the cyclodextrin is added to the mobile phase, and the solute retention is split into two main physicochemical processes, i.e. solute complexation by cyclodextrin and transfer of free (i.e. uncomplexed) solute from the mobile to the stationary phase [13–40]. The association constant K between compound and cyclodextrin can be determined using the well known equation [26,27]:

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K[\mathrm{CD}]^x}{k_0} \quad (1)$$

where k is the solute retention factor, k_0 the solute retention factor without cyclodextrin in the mobile phase [CD] the cyclodextrin concentration and x the stoichiometry of the complex. For an inclusion complex with a 1:1 stoichiometry ($x = 1$) and when the retention of CD-solute compex is negligible, a linear plot of $1/k$ versus [CD] must be obtained and the K value calculated. CDs are extensively used as stationary phase components in gas chromatography as well as stationary or mobile phase components in liquid chromatography [15]. Crini et al. examinated the ability of several β-cyclodextrin (β-CD) bonded stationary phases based on silica beads coated with poly(alkylamine) (poly(ethyleneimine) to separate ortho-, meta- and para-isomers of some disubstitued benzene derivatives [16]. The 8-MOP adsorption isotherms on HP-β-CD bounded stationary phase (i.e. association constant) can be also determined using the perturbation (PT) technique, originally developed for measuring gas-adsorbent equilibria [41,44]. In the second time, these association constant values was used to determine the cytotoxicity profile of human keratinocyte cell line (HaCaT) in relation to the complex concentration.

2. Experimental section and theory

2.1. Chromatographic protocol

2.1.1. Apparatus

The chromatographic system consisted of a HPLC Waters pump 501 (Saint Quentin, Yvelines, France), an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a 20 μl sample loop and a Merck 2500 diode array detector (Nogent-sur-Marne, France). An Interchim *R*/2 HP-β-CD column (125 mm × 4 mm i.d.) was used (Montluçon, France). The *R*/2-HP-β-CD was immobilized on amino groups introduced on the silica support via the amination by the poly(alkylamine) (polyethyleneimine) [15]. The column temperature was controlled in an Interchim oven, TM N° 701 (Montluçon, France) for high temperature and an Osi Julabo FT200 cryoimmerser (Elancourt, France) for low temperature. The mobile phase was fixed at 1 ml/min and the wavelength at 254 nm.

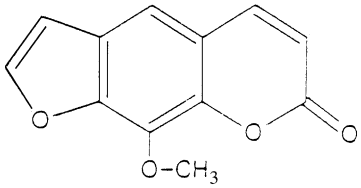

Fig. 1. 8-MOP structure.

2.1.2. Reagents

8-MOP was obtained from Sigma–Aldrich (Saint-Quentin, France) (Fig. 1). Deuterium oxide (Merck, Nogent-sur-Marne, France) was used as a dead time marker. Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The mobile phase consisted of a methanol/water mixture 58–42 (v/v). The equilibration of the column was carried out with nine different concentrations of 8-MOP (0–8 mM) to obtain a stable detection. Twenty microliter of the most concentrated 8-MOP sample was injected three times and the retention times were measured.

2.1.3. Chromatographic theory

The non-linear chromatography determinates the 8-MOP adsorption isotherms using the perturbation technique which consists in the determination of the retention times of small 8-MOP amounts injected onto the column equilibrated with 8-MOP solutions at different concentration levels. The Langmuir theoretical approach relates the total concentration of 8-MOP in the *R*/2-hydroxypropyl-β-cyclodextrin stationary phase (C_s) and that in the mobile phase (C_m) [42–46].

$$C_s = \frac{\alpha K C_m}{1 + K C_m} \quad (2)$$

where α is the column saturation capacity and K is the association constant between 8-MOP and the *R*/2-hydroxypropyl-β-cyclodextrin used as stationary phase. The 8-MOP retention factor k was directly proportional to the slope of its adsorption isotherm and can be thus given by the following equation [45,46].

$$k = \frac{\phi \alpha K}{(1 + K C_m)^2} \quad (3)$$

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase; equal to 0.59 commercial data). By plotting the k values versus the 8-MOP concentrations in the bulk solvent, the association constant K can be determined using Eq. (2). However, it was possible that the 8-MOP could tightly bind to the residual poly(alkylamine (poly(ethyleneimine) of the stationary phase. Then, if the 8-MOP in the sample bound on two sites on the stationary phase, i.e. a specific site (site I: with an adsorption constant K_I, and a column saturation capacity α_I) and a second site which is non-specific (site II; with an adsorption constant K_{II} and a column saturation capacity α_{II}), then the 8-MOP retention factor (k) directly

proportional to the slope of its adsorption isotherm is given by the following equation [44].

$$k = \phi\left(\frac{\alpha_I K_I}{(1+K_I C)^2} + \frac{\alpha_{II} K_{II}}{(1+K_{II}C)^2}\right) = \frac{\bar{k}_I}{(1+K_I C)^2} + \frac{\bar{k}_{II}}{(1+K_{II}C)^2} \quad (4)$$

where $\bar{k}_I$ (equal to $\phi\alpha_I K_I$) and $\bar{k}_{II}$ (equal to $\phi\alpha_{II}K_{II}$) are the apparent retention factors (retention factor when the 8-MOP concentration in the mobile phase was nil) of respectively the 8-MOP association on the stationary phase specific site of high affinity (site I; corresponding to the 8-MOP complexation with the HP-β-CD grafted on the stationary phase) and low affinity (site II; corresponding to the 8-MOP association with the residual amino groups on the particles of the stationary phase). Then, using a non-linear regression analysis, by studying the variation of the k values versus the sample concentration in the mobile phase, the apparent retention factors K_I and K_{II} can be calculated.

2.2. Biological assay

2.2.1. Materials, drugs and chemicals

HaCaT cells were kindly given by Dr N. Gault (CEA, Bruyère Le Chatel, France); Pure, *R*/2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from CEA Fontenay-aux-Roses, France); 8-methoxypsoralen (8-MOP), Dubelcco's Modified Eagle's Minimum Essential Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), Dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France); Costar culture flasks, microtiter plates, fetal calf serum (FCS) and Trypsin were from D. Dutscher (Brumath, France). Phosphate-buffered saline (PBS without calcium and magnesium), Trypsine and HEPES were from VWR International (Cergy-Pontoise, France).

2.2.2. Culture of HaCaT cells [47]

HaCaT cells were routinely grown in 75 cm^2 Costar plastic flasks in monolayer cultures in DMEM medium supplemented with 10% (v/v) FCS, and 5 M of HEPES. They were grown in a humidified atmosphere of 5% CO_2 in air. The medium was renewed routinely 2, 4 and 6 days after passage and when confluence was attained, cells were trypsinized and split for subcultures (seeding density 3500 cells/cm^2 in a 75 cm^2 flask) or used for cytotoxicity assays. Cells were used for experiments within 10 passages to ensure cell line stability. All the experiments were carried out at 25 °C.

2.3. Cytotoxicity assays

2.3.1. Treatment of cultures

HaCaT cells were seeded at a density of 6×10^4 cells per well in 100 μl culture medium containing 10% FCS in 96-well microtiter plates and incubated overnight for adherence. The following day, the medium was discarded and the cells were incubated in FCS-free medium containing increasing concentrations of 8-MOP (with <0.5% ethanol vehicle) and/or HP-β-CD (up to 675 μM) and exposed to a dose of UVA radiation of 2 J/cm^2 (320–400 nm) during 460 s at a tube-to-target distance of 20 cm. Immediately after irradiation, the treated cells were then cultured for an additional 24 h period. Control cells were not exposed to drugs (8-MOP or HP-β-CD).

2.3.2. Cell viability [48]

Cell survival was assayed by measuring mitochondrial activity with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [1]. This assay is based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble blue formazan product. Only viable cells with active mitochondria reduce significant amounts of MTT. After treatment, the reaction medium was removed and the adhering cells were washed with PBS. One hundred microliter MTT solution (0.5 g/l in medium) was added to the culture wells and after a 4 h incubation at 37 °C, the medium/MTT mixture was replaced with 100 μl of DMSO. Blue formazan formation was quantified with a spectrophotometer at 570 nm. Values of absorbance were converted into percentage of residual viability (Y). Usually, inhibition concentration 50% (IC50) was chosen as the best biological marker of cytotoxicity.

3. Results and discussion

For each 8-MOP concentration in the bulk solvent the most concentrated 8-MOP sample was injected and its retention factor was determined. The variation coefficients of the k values were <0.6%, indicating a high reproducibility and a good stability for the chromatographic system. The variation of the k values versus 8-MOP concentration in the bulk solvent was similar for the two studied temperatures (5 and 25 °C). Using a weighted non-linear regression (WNLIN) procedure, the constants of equation 3 were used to estimate the retention factors ($\alpha = 78.4$ at 25 °C). This α value was in accordance with the one reported by Jandera et al. [44]. The slope of the curve representing the variation of the estimated retention factors (Eq. (3)) versus the experimental values (0.999; ideal is 1.000) and r^2 (0.997) indicate that there is an excellent correlation between the predicted and experimental retention factors. From the full regression model, a student's t-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of the student's t-test show that no variables can be excluded from the model. These results showed that the Langmuir model describes accurately the association behaviour of 8-MOP with HP-β-CD. Then, the concentration of the 8 MOP in the stationary phase (C_s) can be evaluated using Eq. (2). The scatchard plot of $1/C_s$

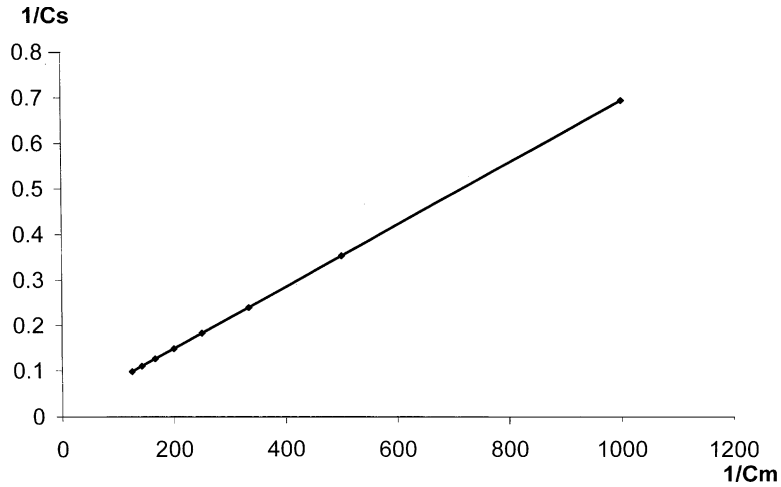

Fig. 2. Scatchard plot of $1/C_s$ (mM) vs. $1/C_m$ (mM) at 25 °C.

versus $1/C_m$ was given in Fig. 2 at 25 °C. The corresponding K values were at 25 and 5 °C, respectively, equal to 18.71 and 29.52. These values were in the same order of magnitude than the one obtained by the classical method (Eq. (1)) for which HP-β-CD was dissolved in the mobile phase (at 5 °C, $K = 30.11\,M^{-1}$). This confirmed that bonded HP-β-CD on silicea do not modified the inclusion process. As well, this is consistent with previous results who have shown by spectroscopic or chromatographic methods the formation of inclusion complex between psoralen with β-CD, dimethyl DM-β-CD and trimethyl TM-β-CD [50,51].The principal disadvantage of this classical method was that HP-β-CD was no a pure compound but rather mixture of homologous and isomers [49]. In this study, this disadvantage was cancelled, and the "real" association constant could be therefore determined between 8-MOP and *R*/2-HP-β-CD. However, the immobilization of HP-β-CD on silica support could led to a non-specific interaction, i.e. association of the 8-MOP with the residual amino group of the chromatographic support (see chromatographic protocol). Then, using a non-linear regression, the K_I and K_{II} values (i.e. the adsorption constant of the 8-MOP, respectively, with the high affinity site (specific interaction) and with the low affinity site (non-specific interaction) were determined from Eq. (4) (at 25 °C, $\alpha_I = 78.3$, $\alpha_{II} = 1.2$). The non-linear coefficient results ($r^2 > 0.99$) proved that the two-order Langmuir model described accurently the binding mechanism of 8-MOP with the stationary phase. The results showed that the interactions between the 8-MOP and the residual amino groups of the stationary phase were neglected (at 5 °C $K_{II} = 0.11\,M^{-1}$ and at 25 °C $K_{II} = 0.09\,M^{-1}$). This confirmed that the preparation of HP-β-CD bounded phase column via amination of silica by poly(alkylamine) (poly(ethyleneimine) was good [3]. As expected the K_I values were similar to the one obtained with Eq. (3) (at 25 °C $K_I = 18.65\,M^{-1}$ and at 5 °C $K_I = 28.66\,M^{-1}$).

Then, the effect of the complexation of 8-MOP with *R*/2-HP-β-CD on a human keratinocyte cell line was eval-

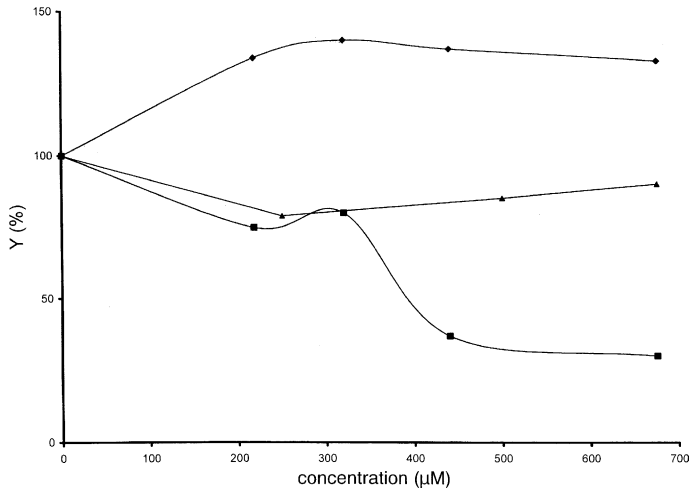

Fig. 3. Influence of UVA light (2 J/cm^2, 460 s) on 8-MOP cytotoxicity profile ((◆) without UVA; (■) with UVA) in cultured HaCaT cells after 24 h of treatment and cytotoxicity profiles of HP-β-CD alone (▲) under UVA light, in cultured HaCaT cells after 24 h of treatment.

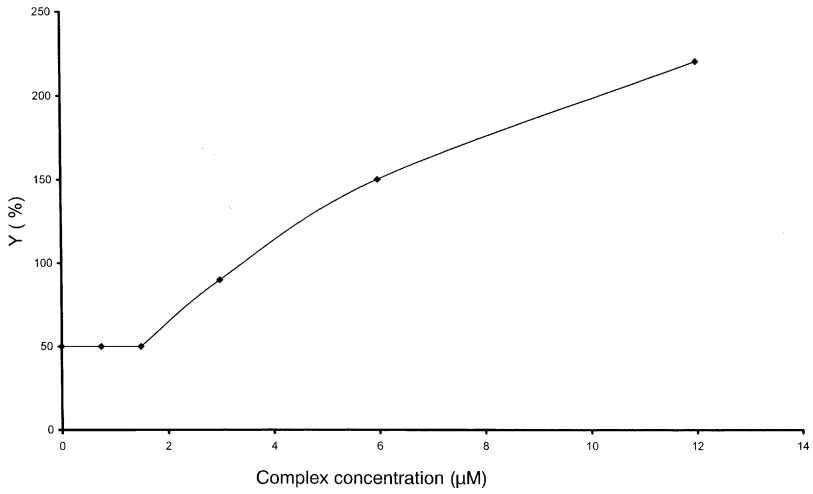

Fig. 4. Variations of *Y* (percentage of residual viability) in relation to the complex concentration (with 8-MOP concentration equal to 400 μM) in cultured HaCaT cells after 24 h of treatment.

uated. In preliminary assays, it was shown that neither 8-MOP (Fig. 3: Y (percentage of residual viability) up to 100%) nor UVA radiation (for this last physical parameter *Y* was constant equal to around 100%) altered the cell viability of HaCaT culture. On the contrary, at all concentrations tested, 8-MOP plus UVA radiation was toxic (Fig. 3). The 8-MOP cytotoxicity profile was similar as a sigmoid where percentage of residual cell viability (Y) depends to psoralen amounts : the highest concentrations (up to 500 μM) were very cytotoxic ($Y < 30\%$) and the inhibition concentration 50% (IC50) amounted to 400 μM. These data are in agreement with other reports which demonstrated that PUVA (8-MOP/UVA) treatment of a mouse epidermal cell line [13] or human mononuclear cells [52] caused cell death while neither 8-MOP nor UVA alone had a cytotoxic effect. Moreover, it appeared that the *R*/2-HP-β-CD, from 200 to 700 μM, had not cytotoxic effect on HaCaT (Fig. 3). The 8-MOP association with HP-β-CD in the mobile phase was represented by the following equation:

$$\text{8-MOP} + \text{HP-β-CD} \leftrightarrow \text{8-MOP/HP-}\beta\text{-CD} \quad (5)$$

For an inclusion complex with a 1:1 stoichiometry, the equilibrium constant *K* of Eq. (5) is as follows [25–28]:

$$K = \frac{[\text{8-MOP/HP-β-CD}]}{[\text{8-MOP}][\text{HP-β-CD}]} \quad (6)$$

where [8-MOP/HP-β-CD], [HP-β-CD] and [8-MOP] are the complex, HP-β-CD and 8-MOP concentration, respectively. As *K* was previously determined (equal to 18.71 M^{-1}) by the chromatographic approach, Eq. (6) allowed to rely the cytotoxicity with the complex concentration value. Fig. 4 presents the cytoxicity profile in relation to the 8-MOP/HP-β-CD concentration (i.e. complex concentration) when the 8-MOP concentration was equal to 400 μM (CI50). As shown in Fig. 4, when the complex concentration increased, the toxicity decreased. Interestingly, it was observed that from 1.4 to 11.9 μM of 8-MOP/HP-β-CD concentration, complexation of 8-MOP with the cyclodextrin noticeably reduced the toxicity of 8-MOP on HaCaT cells and showed a cytoprotection.

4. Conclusion

This paper described the 8-MOP/(*R*/2-HP-β-CD) association using a new chromatographic methodology. The results obtained confirmed the possible association of 8-MOP with *R*/2-HP-β-CD. The apparent association constants were determined experimentally for a temperature equal to 25 °C and at a very low temperatures (5 °C). The association constants values were respectively equal to 18.7 and 29.5. As well these data clearly demonstrated that *R*/2-HP-β-CD plays an important role in the cell protection towards toxicity of 8-MOP. This report is to our knowledge the first describing the decrease of 8-MOP/UVA cytotoxicity in vitro in human epidermal cells as keratinocytes by a complexant agent as HP-β-CD. But some further studies have to be thinking of enhancing this protective effect. For instance, the understanding of the complexation phenomenon is necessary to appreciate the role of the complex in the protective effect. The possible formation of inclusion compounds in the case of psoralen derivatives may have pharmacological and analytical implications and utilisation of HP-β-CD as a drug carrier might be of interest for biological and clinical use.

References

[1] H. Hônigsmann, E. Jaschke, F. Gschnait, W. Brenner, P. Frisch, K. Wolff, Br. J. Dermatol. 101 (1979) 369.
[2] P. Muret, S. Makki, S. Urien, P. Bechtel, P. Agache, J.P. Tillement, Pharm. Week 1[Sci] (14[Suppl F]) (1992) 41.
[3] J. Schmidt, R. Brick, U. Busch, F.W. Koss, in: Proceedings of the International Psoralens SIR, Pergamon Press, New York, 1981, pp. 100–146.

[4] L.M.L. Stolk, A.H. Siddiqui, W. Westherhof, R.H. Cormane, Br. J. Dermatol. 112 (1995) 469.
[5] P. Muret, Ph. Humbert, S. Makki, P. Bechtel, S. Urien, J.P. Tillement, J.P. Arch, Dermatol. Res. 287 (1993) 6285.
[6] J.D. Laskin, E. Lee, Biochem. Pharmacol. 41 (1) (1991) 125.
[7] J.D. Laskin, Fd. Chem. Toxic. 32 (2) (1994) 119.
[8] J.D. Laskin, E. Lee, D.L. Laskin, M.A. Gallo, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 8213.
[9] K. Punnonnen, C.T. Jansen, A. Puntala, M. Ahotupa, Soc. Invest. Dermatol. 96 (1991) 255.
[10] F. Aubin, C.K. Donawho, L. Kripke, Cancer Res. 51 (1991a) 5893.
[11] F. Aubin, L. Kripke, S.E. Ullrich, Soc. Invest. Dermatol. 97 (1991) 995.
[12] J.D. Alaskan, E. Lee, E.J. Yurkow, D.L. Laskin, M.A. Gallo, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 6158.
[13] A.B. Santamaria, D.W. Davis, D.X. Nghiem, D.J. McConkey, S.E. Ullrich, M. Kapoor, G. Lozano, H.N. Ananthaswamy, Cell Death Differ. 9 (2202) 549.
[14] M. Otagiri, T. Imai, K. Uekama, J. Pharmacobiodyn. 12 (1982) 1027.
[15] J. Szejtli, Cyclodextrins and their Inclusion Complexes, Academia Kiado, Budapest, Hungary, 1982.
[16] G. Crini, Y. Lekchiri, M. Morcellet, Chromatographia 40 (1995) 296.
[17] A.R. Bary, I.G. Tucker, N.M. Davies, Eur. J. Pharm. Biopharm. 50 (2000) 237.
[18] M.S. Nagarsenker, R.N. Meshram, G. Ramprakash, J. Pharm. Pharmacol. 52 (2000) 949.
[19] B. Cappelo, C. Carmignani, M. Iervolino, M. Immacolata La Rotonda, M.F. Saettone, Int. J. Pharm. 213 (2001) 75.
[20] M.L. Vasquez, C.M. Franco, A. Cepeda, P. Prognon, G. Mahuzier, Anal. Chim. Acta 269 (1992) 239.
[21] S. Hamai, J. Phys. Chem. 93 (1989) 2074.
[22] K.A. Connors, Binding Constants: a measure of Molecular Complex Stability, Wiley, New York, 1987.
[23] P.W. Carr, J.M. Harris, Anal. Chem. 58 (1986) 626.
[24] A. Munoz de la Pena, T.T. Ndou, J.B. Zung, I.M. Warner, J. Phys. Chem. 63 (1991) 1018.
[25] Y.C. Guillaume, E. Peyrin, Anal. Chem. 71 (1999) 2046.
[26] K. Shimada, K. Mitamura, M. Muira, A. Miyamoto, J. Liq. Chromatogr. 18 (1995) 2885.
[27] E. Peyrin, Y.C. Guillaume, Chromatographia 49 (1999) 691.
[28] N. Morin, Y.C. Guillaume, E. Peyrin, J.C. Rouland, J. Chromatogr. A 808 (1998) 51.
[29] K.H. Lamparczyk, P.K. Zarzycki, J. Pharm. Biomed. Anal. 13 (1995) 543.
[30] S. Shuang, M.M. Choi, J. Chromatogr. A 919 (2001) 321.
[31] V.C. Anigbogu, A. Munoz de la Pena, T. Ndou, I.M. Warner, Anal. Chem. 64 (1992) 484.
[32] N. Husain, V.C. Anigbogu, M.R. Cohen, I.M. Warner, J. Chromatogr. 635 (1993) 211.
[33] B. Sebille, N. Thuaud, J. Piquion, N. Behar, J. Chromatogr. 409 (1987) 61.
[34] S.El. Gezawi, N. Omar, N.El. Rabbat, H. Ueda, J.H. Perrin, J. Pharm. Biomed. Anal. 6 (1988) 399.
[35] A.M. Stalcup, S.C. Chang, D.W. Armstrong, J. Pitha, J. Chromatogr. 513 (1990) 181.
[36] Y. Tang, J. Zukowski, D.W. Armstrong, J. Chromatogr. A 743 (1996) 261.
[37] D.W. Armstrong, W. De Mond, J. Chromatogr. Sci. 22 (1984) 411.
[38] D.W. Armstrong, W. de Mond, P. Czeh, Bronislaw 57 (1985) 481.
[39] R.E. Boehm, D.E. Martire, D.W. Armstrong, Anal. Chem. 60 (1988) 522.
[40] A. Berthod, H.L. Jin, T.E. Beesley, J.D. Duncan, D.W. Armstrong, J. Pharm. Biomed. Anal. 8 (1990) 123.
[41] D.W. Armstrong, L.A. Spino, S. M Han, I. Seeman, H.V. Secor, J. Chromatogr. 411 (1987) 490.
[42] D.W. Armstrong, Anal. Chem. 59 (1987) 84A.
[43] G. Guiochon, S. Golshan-Shirazi, A.M. Katti, Fundamentals of Preparative and Nonlinear Chromatography, Academic Press, Boston, 1994.
[44] P. Jandera, S. Buncekova, K. Mihlbachler, G. Guiochon, V. Backovska, J. Planeta, J. Chromatogr. A 925 (2001) 19.
[45] C. André, Y.C. Guillaume, Chromatographia 52 (2000) 753.
[46] C. Blumel, P. Hugo, A. Seidel-Morgenstern, J. Chromatogr. 265 (1999) 51.
[47] P. Boukamp, R.T. Petrussevska, D. Breitkreutz, J. Harnung, A. Markham, R.E. Fusenig, J. Cell Biol. 106 (1988) 761.
[48] T. Mosmann, J. Immunol. Meth. 65 (1983) 55.
[49] L. Ismaili, C. André, L. Nicod, J.L. Mozer, J. Millet, B. Refouvelet, S. Makki, J.F. Robert, A. Xicluna, Y.C. Guillaume, J. Liq. Chromatogr. Related Technol. 26 (2003) 871.
[50] F.F. Vincieri, G. Mazzi, N. Mullinaci, M. Bambagiotti-Alberti, F. Dall'Acqua, D. Vedaldi, Farmaco (Soc. Chim. Italia 1989) 50 (7–8) (1995) 543.
[51] J. Blais, P. Prognon, G. Mahuzier, P. Vigny, J. Photochem. Photobiol. B 2 (4) (1998) 455.
[52] G. Klosner, F. Trautinger, R. Knobler, P. Neuner, Soc. Invest. Dermatol. 116 (2001) 459.