ELSEVIER

Journal of Chromatography A, 924 (2001) 483–491

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of apparent binding constants of drugs by capillary electrophoresis using β-cyclodextrin as ligand and three different linear plotting methods

Maria Silvana Bellini[a,*], Zdenek Deyl[b], Giulia Manetto[c], Michaela Kohlíčková[d]

[a]*GlaxoSmithKline, Medicines Research Centre, Via Fleming 4, Verona, Italy*
[b]*Institute of Physiology, Academy of Sciences of Prague, Prague, Czech Republic*
[c]*Department of Public Medicines and Health, University of Verona, Verona, Italy*
[d]*Department of Analytical Chemistry, Prague Institute of Chemical Technology, Prague, Czech Republic*

Abstract

Capillary electrophoretic estimation of apparent binding constants (K_{app}) for naproxen, salbutamol, indomethacine and procaine with β-cyclodextrin is presented. While with naproxen and indomethacine this approach was straightforward and gave well compatible results by three different linearization plots (double reciprocal, *x* reciprocal and *y* reciprocal), with salbutamol a higher value than reported for the electromigration estimation of this magnitude was obtained (a fourfold increase). This difference is ascribed to the fact that the measurements were done in the acid region (while the reported values were obtained at higher pH values). As a matter of fact the values of K_{app} reported in this communication for salbutamol comply better with the value of K_{app} (69.3) obtained by the solubility method.

Keywords: Binding constants; Pharmaceutical analysis; Complexation; Cyclodextrins; Naproxen; Salbutamol; Indomethacine; Procaine

1. Introduction

Molecular binding is widely characterized by apparent equilibrium constants. A number of approaches have been used for this purpose: literature is abundant with spectroscopic techniques [1,2], separation methods [3–5], calorimetry [6–8], potentiometry and reaction kinetics (for reviews see Refs. [9,10]). A relatively recent technique that falls into the category of separation methods and exploits the changes in the electrophoretic mobility of the complexed solute in the background electrolyte containing the complexation agent (ligand) is capillary electrophoresis (CE). This topic has been recently reviewed in depth by Rundlett and Armstrong [10].

In order to be able to apply CE for this purpose the following conditions must be satisfied: (i) the solute must exhibit a mobility change upon complexation; (ii) the time needed to reach the equilibrium must be much faster than the running time during the electrophoretic experiment and (iii) sufficient concentrations of both the solute and the ligand must be available in the system [11,12]. In pharmaceutical chemistry the most widely used ligands are cyclodextrins which are capable of complexing

*Corresponding author.

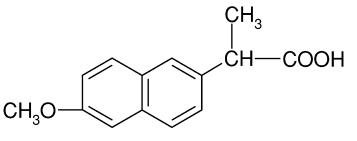
0021-9673/01/$ – see front matter
PII: S0021-9673(01)00775-0

hydrophobic compounds increasing thereby the stability and aqueous solubility of a number of drugs [13–15]. Though a number of cyclodextrins and their derivatives has been investigated from this point of view, the most generally used complexing agent today is β-cyclodextrin (β-CD). An exhaustive review on this subject has been published recently [19].

From the practical point of view a serious hindrance to be circumvented in the electromigration assessment of apparent association constants of β-CD is the limited solubility of the complexing agent in aqueous buffers. Because of this fact direct calculation of the apparent association constant by the mobility difference method and nonlinear curve fitting to CE binding isotherm cannot be applied [16,17].

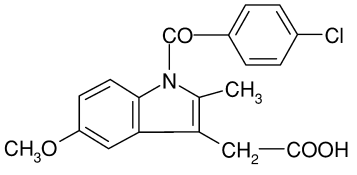
Linear plotting methods represent obviously the most convenient approach provided that the data are properly weighted for linear plotting. A standard Scatchard plot brings about the problem of using the dependent variable on both axes of the plot which complicates statistical evaluation of the data [17,18] and because of this fact it was criticised as producing some degree of inevitable correlation [3]. With the double reciprocal plot the calculation emphasizes the data obtained at the low ligand concentration and was shown to mask deviations from linearity [3].

In this communication we attempted to obtain apparent association constants of four model drugs (salbutamol, naproxen, procaine and indomethacine, Fig. 1) with β-CD by CE and to compare the results obtained by three linearization methods, namely the double reciprocal, *y* reciprocal and *x* reciprocal plots.



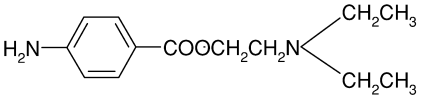
NAPROXEN

Rel. mol. mass = 230.26 g/mol



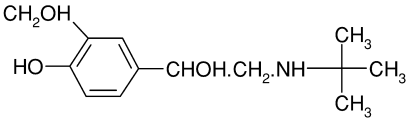
INDOMETHACIN

Rel. mol. mass = 357.81 g/mol



PROCAINE

Rel. mol. mass = 236.30 g/mol



SALBUTAMOL

Rel. mol. mass = 239.31 g/mol

Fig. 1. Structures of the model drugs tested. For apparent binding constants obtained during this experimental work see Table 2.

2. Theory

If the solute (S) binds to a ligand (L) in a 1:1 ratio then the system can be described by the following equation:

$$S + L = SL$$

The electrophoretic mobility (μ) of the solute in a background electrolyte containing the ligand is the weighted average of the complexed (m_c) and uncomplexed (m_f) solute:

$$m_i = X_f m_f + X_c m_c \quad (1)$$

where m_i is the experimentally measured mobility and X represents the molar fractions of the solute in the free (X_f) and complexed (X_c) state.

Introducing equilibrium concentrations the following equation is obtained:

$$m_i = \frac{[S]}{[S]+[SL]} m_f + \frac{[SL]}{[S]+[SL]} m_c \quad (2)$$

Using the expression of equilibrium constant for the considered case:

$$K = \frac{[SL]}{[S]_f [L]_f} \quad (3)$$

and combining this expression with Eq. (2), the following equation for the solute mobility at any given concentration of the complexing agent in the background electrolyte is obtained:

$$K[L] = \frac{(m_f - m_i)}{(m_i - m_c)} \quad (4)$$

from which it follows that:

$$m_i = \frac{m_f + m_c K[L]}{1 + K[L]} \quad (5)$$

Evidently the constant K obtained in this way refers to concentrations and is not the true thermodynamic equilibrium constant [10].

By rearranging this expression (for details see Refs. [3,9]) the following possibilities are available for linearization:

(A):

$$\frac{1}{(m_i - m_f)} = \frac{1}{(m_c - m_f)K} \cdot \frac{1}{[L]} + \frac{1}{(m_c - m_f)}$$

This leads to a double reciprocal plot of $1/(m_i - m_f)$ vs. $1/[L]$ and the apparent binding constant K = intercept/slope:

(B):

$$\frac{[L]}{(m_i - m_f)} = \frac{1}{(m_c - m_f)} \cdot [L] + \frac{1}{(m_c - m_f)K}$$

This leads to y reciprocal plot of $[L]/(m_i - m_f)$ vs. $[L]$ and the apparent binding constant K = slope/intercept, and (C):

$$\frac{(m_i - m_f)}{[L]} = -K(m_i - m_f) + K(m_c - m_f)$$

This leads to the x reciprocal plot of $(m_i - m_f)/[L]$ vs. $(m_i - m_f)$ and the apparent binding constant (K_{app}) equals $-$slope.

3. Methods

3.1. Capillary electrophoresis

All separation methods were done in an untreated fused-silica capillary [32 cm (23 cm to the detector) × 75 μm I.D.] purchased from Composite Metal Services (Hallow, UK) mounted in a HP ^{3D}CE electropherograph (Agilent Technologies, Cernusco sul Naviglio, Milan, Italy). Detection was by UV absorbance at 200 nm.

In between individual runs the capillary was rinsed step-wise by water (1 min), 1.0 *M* NaOH (1 min), 0.1 *M* NaOH (1 min), water (1 min) and the run buffer (6 min). Dimethyl sulfoxide (DMSO) served as an endoosmotic flow marker and was added to the sample at a concentration of 1 μg/ml.

Separation conditions are specified in Table 1.

3.2. Chemicals

Drugs tested were of the following provenience: salbutamol (as analytical standard by Glaxo SmithKline, Verona, Italy), naproxen, procaine and indomethacine (Sigma–Aldrich, Milan, Italy). Before application the drugs were dissolved in run buffer at a concentration of about 150 μg/ml.

All other chemicals were of analytical-reagent

Table 1
Experimental conditions for the capillary electrophoresis for the estimation of the apparent binding constants of the four drugs investigated. All runs at 25°C, capillary length 32/23 (to the detector), I.D. 75 μm

Drug	Concentration range of β-CD investigated (mmol/l)	Injection (mbar/s)	Voltage (kV)	Detection, UV (nm)	Buffer used
Naproxen	0–3	20/3	25	200	10 m*M* phosphate, pH 5.5
Indomethacine	0–3	35/3	10	200	10 m*M* phosphate, pH 5.5
Procaine	0–5	20/3	5	200 and 230	10 m*M* phosphate, pH 5.5
Salbutamol	0–12	20/3	20	200	40 m*M* phosphate, pH 3.0

Concentrations of the drugs in samples injected: Naproxen 144 μg/ml, Indomethacine 71 μg/ml, Procaine 143 μg/ml, Salbutamol 143 μg/ml. Rinse cycles: water, 1.0 *M* NaOH, 0.1 *M* NaOH, water; 1 min each followed by 6 min rinse with run buffer.

grade or highest available purity and were purchased from Sigma–Aldrich.

3.3. Data evaluation

The mobility data plots were calculated using the Microcal Origin version 4.10 program (Microcal Software, Northampton, MA, USA).

If not stated otherwise all cyclodextrin concentrations are in mmol/l and mobilities (mobility differences) in $cm^2/V\,s$.

4. Results and discussion

Data for the estimation of the β-CD–naproxen, –indomethacine, –procaine and –salbutamol apparent binding constants are presented in Tables 2–4

Table 2
Apparent binding constants (K_{app}) and pK_{app} for the β-CD-naproxen, indomethacine, procaine and salbutamol interaction

	Parameters in the $y = a + bx$ linearization		Apparent binding constant	
	a	*b*	K_{app} (M^{-1})	pK_{app}
Naproxen				
A) double reciprocal fit	14.7586	9.3914	1571	3.19
B) *X*-reciprocal fit	10.8810	12.8455	1180	3.07
C) *Y*-reciprocal fit	0.0994	−1.3492	1349	3.13
Indomethacine				
A) double reciprocal fit	4.9223	11.6166	423	2.62
B) *X*-reciprocal fit	11.0034	5.3504	486	2.68
C) *Y*-reciprocal fit	0.0882	−0.4526	452	2.65
Procaine				
A) double reciprocal fit	3.6722	234.1779	15.68	1.19
Salbutamol				
A) double reciprocal fit	−105.9056 (−9.1300)	2317.5568 (0.8947)	45.69 (10.25)	1.65 (1.01)
B) *X*-reciprocal fit	−2232.7014	96.7547	43.33	1.63
C) *Y*-reciprocal fit	$-4.4603\cdot10^{-4}$	0.0437	43.70	1.64

Parameters for the three types of the linearization fits (double reciprocal, *X*- and *Y*-reciprocal) for the four drugs tested. Data in parenthesis for salbutamol refer to 25 m*M* phosphate buffer concentration.

Table 3
Statistical evaluation of the linearization fits for apparent binding constant evaluation (β-CD vs. the drug tested)

	Value	Error	*t*-Value	Prob>|t|
Naproxen				
Double reciprocal				
Parameter a	14.7586	1.7035	8.6632	$1.3045 \cdot 10^{-4}$
Parameter b	9.3914	0.7104	13.2198	<0.0001
X-reciprocal				
Parameter a	10.8810	0.9314	11.6823	<0.0001
Parameter b	12.8455	0.6734	19.0742	<0.0001
Y-reciprocal				
Parameter a	0.09949	0.0085	11.6444	<0.0001
Parameter b	−1.3492	0.2185	−6.1724	$8.3086 \cdot 10^{-4}$
Indomethacine				
Double reciprocal				
Parameter a	4.9223	0.3606	13.6493	$8.5076 \cdot 10^{-4}$
Parameter b	11.6166	0.3666	31.6871	<0.0001
X-reciprocal				
Parameter a	11.0034	0.6783	16.2208	$5.0973 \cdot 10^{-4}$
Parameter b	5.3504	0.3718	14.3878	$7.2775 \cdot 10^{-4}$
Y-reciprocal				
Parameter a	0.0882	0.00463	19.0425	$3.1622 \cdot 10^{-4}$
Parameter b	−0.4526	0.05649	−8.0119	0.0040
Procaine				
Double reciprocal				
Parameter a	43.1903	14.4164	2.9959	0.0302
Parameter b	108.5059	30.0995	3.6049	0.0154
Salbutamol				
Double reciprocal				
Parameter a	−105.9056	32.7904	−3.2297	0.04824
Parameter b	2317.5568	289.4764	8.0060	0.0040
X-reciprocal				
Parameter a	−2232.7014	284.6768	−7.8429	0.0043
Parameter b	16.7547	30.3177	3.1913	0.0496
Y-reciprocal				
Parameter a	−4.4603	$6.3729 \cdot 10^{-5}$	−6.9988	0.0059
Parameter b	0.0437	0.0084	5.1486	0.0142
Double reciprocal[a]				
Parameter a	−9.1300	12.5918	−0.7250	0.4919
Parameter b	0.8947	0.0714	12.5149	<0.0001

[a] Refers to 25 m*M* concentration of the run buffer

and in Figs. 2–6 in graphical form. While with naproxen, indomethacine and salbutamol we were able to obtain reliable data (see Tables 3 and 4) and good linearization with all the three plotting methods applied (double reciprocal, *x* reciprocal and *y* reciprocal), with procaine we obtained more or less acceptable results with the double reciprocal fit only (however even this fit was rather poor from the standpoint of its statistical evaluation as documented in Table 4). The general good agreement between the individual plotting methods used was obtained as well as good agreement with literary data for the apparent binding constants (where available) were obtained.

The reason for the failure in electrophoretic method estimation of the apparent binding constants for β-CD and procaine can have several reasons. The first to be considered comes from the fact that much higher CD concentration should be used for accurately determining an apparent p*K* of the order 1.2.

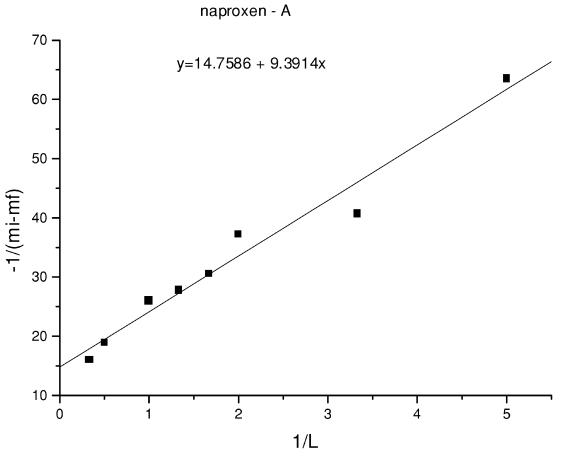
Table 4
R-values for the different linearization fits of apparent binding constant estimation (β-CD vs. naproxen, indomethacine, procaine and salbutamol)

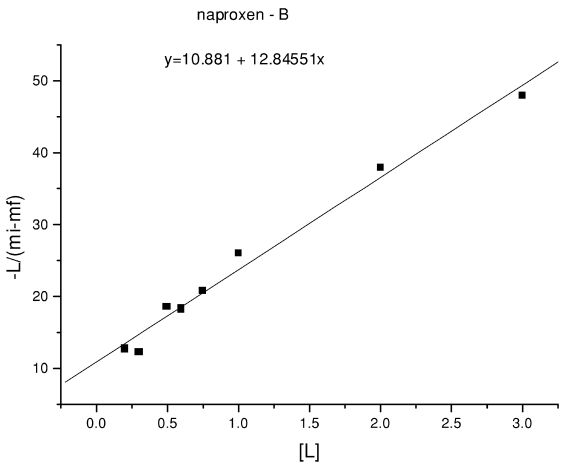
	R	R^2 (COD)	Adj. R^2	Root-MSE (SD)	*N*[a]
Naproxen					
Double reciprocal	0.9832	0.9668	0.9612	2.9528	8
X-reciprocal	0.9918	0.9837	0.9810	1.7284	8
Y-reciprocal	−0.9249	0.8639	0.8412	0.0088	8
Indomethacine					
Double reciprocal	0.9985	0.9970	0.9960	0.4324	5
X-reciprocal	0.9928	0.9857	0.9809	0.6467	5
Y-reciprocal	−0.9774	0.9553	0.9404	0.0030	5
Procaine					
Double reciprocal	0.8497	0.7221	0.6665	19.1403	7
Salbutamol					
Double reciprocal	0.9774	0.9553	0.9404	12.562	5
X-reciprocal	0.8789	0.7725	0.6966	113.2439	5
Y-reciprocal	0.9478	0.8983	0.8644	$4.1409 \cdot 10^5$	5
Double reciprocal[b]	0.9783	0.9572	0.9511	18.1166	9

[a] Refers to averaged points in the plot. Each point represents the average of five.
[b] Refers to 25 m*M* actual runs concentration of the run buffer.

Given the range of β-CD actually used, the measured procaine mobility m_i is too close to its m_f value which leads to poor precision of the term $m_i - m_f$. This also holds for salbutamol, especially for the 25 m*M* phosphate buffer [9]. With naproxen and indomethacine under similar conditions the electrophoretic data offered reliable results. The other point to be considered is the fact that solute mobility can be affected by the ligand in ways other than binding. It was documented that increasing the ligand concentration can change the viscosity of the background electrolyte and its ionic strength thereby changing the electrophoretic mobility, not mentioning the possibility of solute or ligand binding to the capillary wall. This also allows one to conclude that the basic conditions for the apparent binding constant estimation, i.e., that the solute must undergo a change in electrophoretic mobility upon complexation, that the equilibrium for the complex formation must be much faster than the separation and that sufficient concentrations of free ligand and ligand–solute complex must be available were met in our experiments.

A few words have to be said about salbutamol. With 25 mmol/l phosphate concentration in the background electrolyte we obtained the K_{app} value of 10.25 as compared to the reported 9.6 [19] obtained also by an electromigration method. Our calculation refers to the double reciprocal plot only as the other plotting methods gave unusable results (Fig. 5, Table 2). On the other hand, by increasing the concentration of the background electrolyte to 40 mmol/l, we obtained considerably less scattered data which could be subjected to all the linearization plots used. However the estimated K_{app} values obtained by the three different linearization plots applied ranged from 43.33 to 45.69. Two factors have to be mentioned to explain this difference. First, changing the concentration of the background electrolyte leads to a change in viscosity of the background electrolyte, for which no corrections were introduced (compare Ref. [18]). Secondly the reported value of K_{app}, 9.6, was determined in alkaline buffers. Though it was claimed that pH change in the alkaline region where salbutamol acts as a weak acid, has little, if any, effect upon the K_{app} value (however no change in the ionic strength of the buffer was investigated), no information is available about the K_{app} value in the acid media where salbutamol behaves as a weak base. Perhaps, as indicated in the Ref. [13], because hydrophobic interactions play a role in β-CD complexation with salbutamol, it may be expected that at the acid pH used these interactions prevail and, consequently, cause a change (increase) in the K_{app}





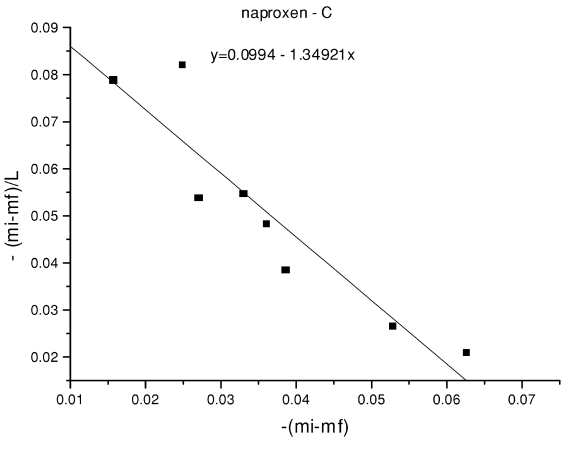
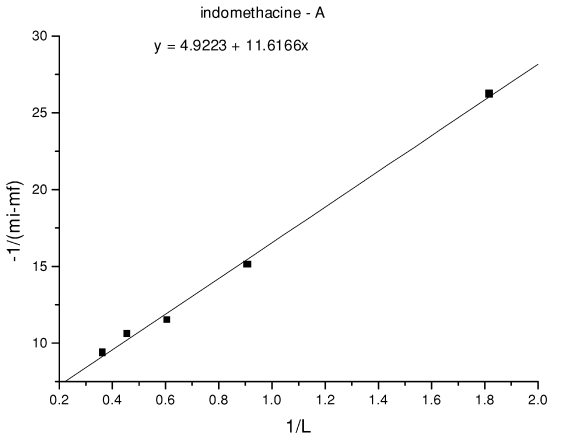
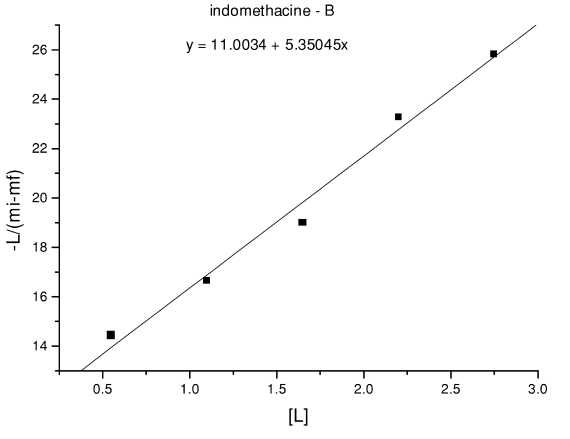


Fig. 2. Comparison of the three different ways of linearization of the mobility data for naproxen: (A) double reciprocal plot; (B) *x* reciprocal plot; (C) *y* reciprocal plot.





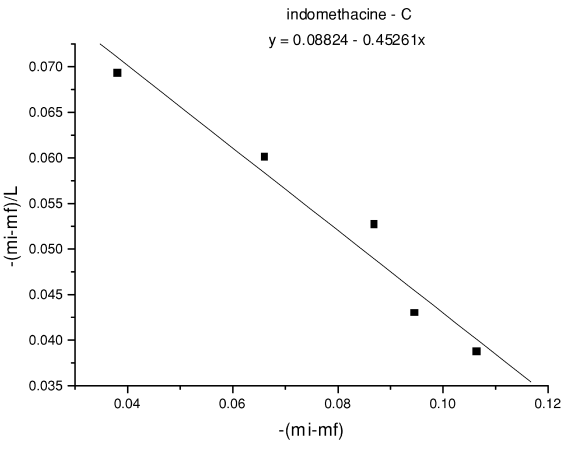


Fig. 3. Comparison of the three different ways of linearization of the mobility data for indomethacine: (A) double reciprocal plot; (B) *x* reciprocal plot; (C) *y* reciprocal plot.

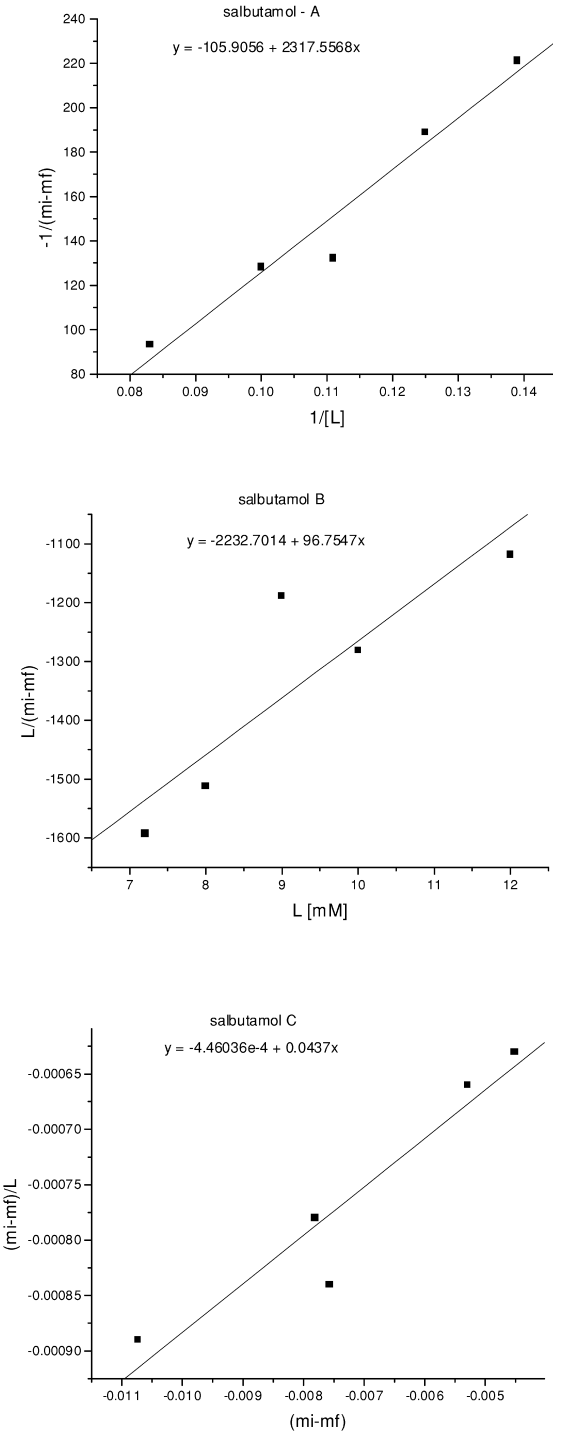


Fig. 4. Comparison of the three different ways of linearization of the mobility data for salbutamol: (A) double reciprocal plot; (B) *x* reciprocal plot; (C) *y* reciprocal plot.

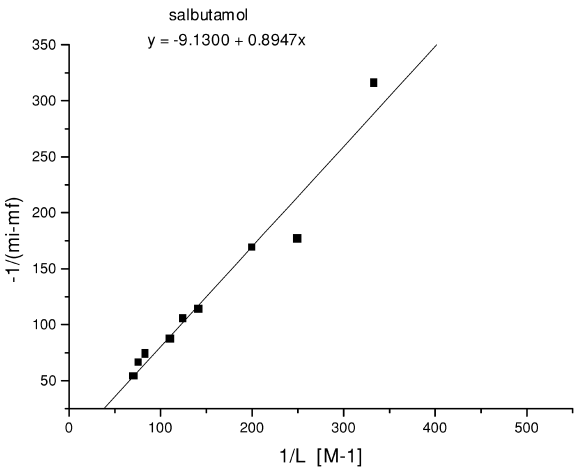


Fig. 5. Double reciprocal linearization of the mobility data for salbutamol obtained at 25 mmol/l buffer concentration.

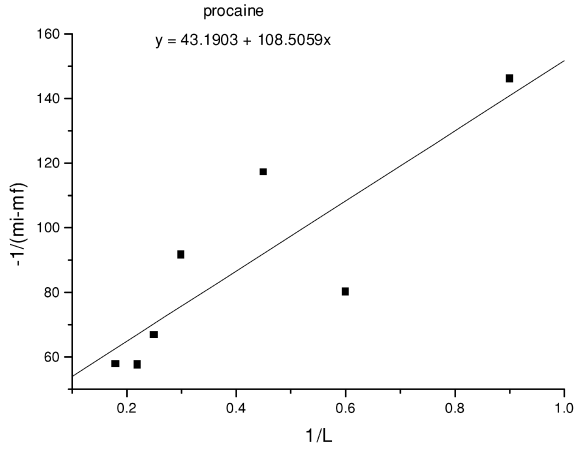


Fig. 6. Double reciprocal linearization of the mobility data for procaine.

value (for detailed discussion of the complexation of uncharged analytes see Ref. [19]). Regarding procaine we failed to find conditions that could offer reliable K_{app} estimation.

Finally using solubility data the K_{app} constant for the salbutamol–β-CD interaction was reported to be 69.3 (see Refs. [20,21]) which is relatively close to our results taking into consideration that the actual numbers were obtained by two completely different techniques.

5. Conclusions

(1) In a particular experimental set-up simultaneous evaluation of the migration data obtained by three different linearization plots (double reciprocal, *x* reciprocal and *y* reciprocal) represents a method that yields reliable K_{app} estimation.

(2) Care must be taken about background electrolyte concentration and the pH of the system used. Both appear to affect the β-CD–drug association considerably, though in a well reproducible manner. The electrophoretic conditions must be carefully optimized for a particular drug–β-CD interaction assay.

References

[1] R. Mathur, E.D. Becker, R.B. Bradley, N.C. Cui, J. Phys. Chem. 67 (1963) 2190.
[2] M.W. Hanna, A.L. Ashbaugh, J. Phys. Chem. 68 (1964) 811.
[3] K.L. Rundlett, D.W. Armstrong, J. Chromatogr. A 721 (1995) 173.
[4] S.A. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[5] A. Guttman, A. Saubus, A. Cohen, N. Grinberg, B.L. Karger, J. Chromatogr. 448 (1998) 41.
[6] G.L. Bertrand, J.R. Faulkner Jr., S.M. Man, D.W. Armstrong, J. Phys. Chem. 93 (1989) 6863.
[7] D. Neerinck, A. Van Audenhague, L. Lamberts, P. Huyskens, Nature 218 (1968) 461.
[8] T.F. Boles, R.S. Drago, J. Am. Chem. Soc. 88 (1966) 3921.
[9] M.V. Rekharsky, Y. Inoue, Chem. Rev. 98 (1998) 1875.
[10] K.L. Rundlett, D.W. Armstrong, Electrophoresis 18 (1997) 2194, and references cited therein.
[11] F. Leliévre, P. Gareil, J. Chromatogr. A 735 (1996) 311.
[12] E. Szökö, J. Gyimesi, L. Barcsa, K. Magyar, J. Chromatogr. A 745 (1996) 181.
[13] V. Lemesle-Lamache, M. Taverna, D. Wouessidjewe, D. Duchéne, D. Ferrier, J. Chromatogr. A 735 (1996) 321.
[14] J. Szejtli, Pharm. Tech. (1991) 15.
[15] M.-Q. Zhang, D.C. Rees, Exp. Opin. Ther. Pat. 9 (1999) 1697.
[16] K.A. Connors, Binding Constants: The Measurement of Molecular Complex Stability, Wiley, New York, 1987.
[17] G.L. Atkins, I.A. Nimms, Anal. Biochem. 104 (1980) 1.
[18] J.L. Carpenter, P. Camilleri, D. Dhanale, D. Goodall, J. Chem. Soc., Chem. Commun. (1992) 804.
[19] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[20] D.O. Thompson, Crit. Rev. Ther. Drug Carrier Syst. 14 (1997) 1.
[21] C. Marques, H.M. Hadgraft, I.W. Kellaway, Int. J. Pharm. 63 (1990) 259.