AN APPROACH TO THE STUDY OF PROTEIN–DRUG INTERACTIONS BY USING CAPILLARY ISOTACHOPHORESIS

Jorgen SJÖDAHL and Sven-Göran HJALMARSSON
LKB-Produkter AB, Research Department, S-161 25 Bromma, Sweden

Received 7 June 1978

1. Introduction

Analytical isotachophoresis in a capillary tube is a highly potent tool for the separation of compounds exhibiting very small differences in net electrophoretic mobility. The basic principles of isotachophoresis have been previously described by several authors [1–3].

It was anticipated, that upon complex formation of a protein and a drug, it should be possible to distinguish between the complex and the native protein by using analytical isotachophoresis. Furthermore, this method seemed feasible for separating and quantitating the non-complexed drug.

The interacting system studied in this work is composed of the protein human serum albumin (HSA) and the anti-inflammatory drug indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid]. This drug is frequently used for the treatment of rheumatic diseases. The molecular formula is shown in fig.1.

The binding of indomethacin (I) to human plasma has been studied by Hvidberg et al. [4], who found

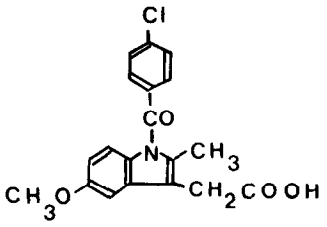

Fig.1. Molecular formula of the drug indomethacin.

fifteen binding sites for the drug, all of which were proposed to be located on the albumin molecule. However, depending on the experimental technique used, Hultmark et al. [5] found indomethacin to bind to four or five binding sites on the HSA-molecule.

The HSA-I system was chosen for this first approach of using analytical isotachophoresis in protein-drug complex formation studies, since it is a relatively-well studied system involving a strongly protein-bound drug.

2. Materials and methods

The human serum albumin and indomethacin were obtained from AB Kabi, Stockholm and A.-S. Dumex, Copenhagen, respectively. The protein was dissolved in distilled water to a concentration of 85 μg/μl (1.3 nmol/μl). Indomethacin was dissolved in the leading electrolyte (see below) to a concentration of 0.65 μg/μl (1.8 nmol/μl).

Incubation mixtures were prepared from these two stock solutions according to table 1. The samples were incubated at ambient temperature for one hour before analysis.

The analyses were performed with a LKB 2127 Tachophor (LKB-Produkter AB, Sweden) equipped with a 23 cm capillary tubing. The leading electrolyte was 5 mM HCl, 10 mM 2-amino-2-methyl-1,3-propanediol (ammediol, Sigma Chem. Co., USA) with a 0.4% HPMC (hydroxypropylmethylcellulose, Methocel 90 HG, 15 000 cps, Dow Chem. Co., USA), pH 9.0. Chloride acts as the leading ion and ammediol as the counter ion, and HPMC was added to counter-

Table 1
Composition of incubated samples

Sample (Refers to fig.3)	Volume albumin solution (μl)	Volume indomethacin solution (μl)	Volume buffer (μl)	Injected volume (μl)	Injected amount of Albumin (nmol)	Albumin (μg)	Indomethacin (nmol)	Indomethacin (μg)	Molar ratio of albumin to indomethacin (HSA:I)
a	50	25	175	5	1.3	85	0.9	0.33	1 : 0.7
b	50	50	150	5	1.3	85	1.8	0.65	1 . 1.4
c	50	100	100	5	1.3	85	3.6	1.3	1 : 2.8
d	50	200	–	5	1.3	85	7.2	2.6	1 : 5.5

Experimental details are given in section 2

act electroendosmosis. The terminating electrolyte was 5 mM ε-aminocaproic acid (Roth, Karlsruhe) adjusted to a pH of about 10.5 with freshly made $Ba(OH)_2$ solution (Merck, FRG). The separations were performed at 12°C with a constant current of initially 170 μA and of 68 μA during detection.

Due to the high UV-absorptivity of the protein as well as of the drug, the standard UV-detector (254 nm) was used. The samples were injected in volumes of 2.5 or 5 μl.

3. Results and discussion

Figure 2 illustrates the UV-profiles obtained from the analyses of the individual components of the incubation mixtures. It is evident that a very pure preparation of HSA was used, as indicated by the homogeneity and the very sharp zone boundaries (fig.2a). Furthermore, indomethacin with its strongly UV-absorbing chromophore appears as a homogeneous and well-defined UV-profile (fig.2b). The small UV-absorbing spikes, as well as the shoulder on the indomethacin peak, were also observed when analyzing a 'blank'. Consequently, they were ascribed to impurities in the electrolyte system. From this figure, it can also be concluded that indomethacin gives a higher UV-absorptivity than albumin at isotachophoretic concentrations under the experimental conditions used.

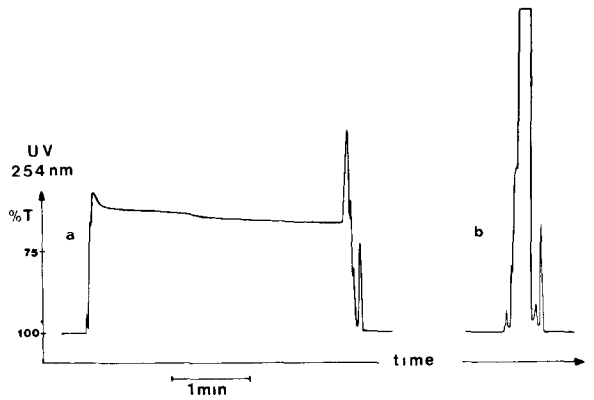

Fig.2. UV-profiles from the isotachophoretic analysis of albumin (fig.a) and indomethacin (fig.b) respectively. Experimental details are given in section 2. The injected amounts were 210 μg and 0.81 μg of albumin and indomethacin, respectively.

The titration of the binding sites on HSA with indomethacin is illustrated in fig.3. It should be stressed that, in isotachophoretic analysis, the zone-width and not the zone-height reflects the amount of material in each zone. Comparing the zones mutually and to the zone of free albumin in fig.2a (1.3 and 3.3 nmol respectively was injected), it is evident that the relative zone-width changes during the titration. This may reflect a change in net electrophoretic mobility when the drug binds to the albumin molecule. However, the significant feature of these UV-profiles is the increase in peak-height when increasing the amount of indomethacin. This reflects the difference beween the UV-absorptivity of HSA alone and that of the complex of HSA-I_n, where n indicates the relative molar amount of bound indomethacin. Furthermore, it is evident that no or very small amounts of free indomethacin are present in the first three samples. It is concluded that, for these molar ratios, the binding of the drug to the protein is practically

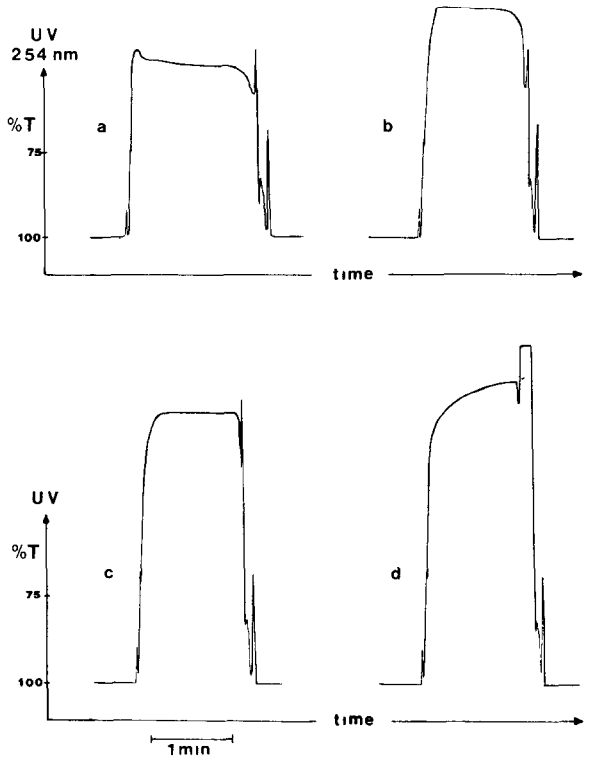

Fig.3. UV-profiles from the isotachophoretic analysis of incubation mixtures containing various ratios of albumin to indomethacin. Experimental details are given in section 2. Ratio of albumin to indomethacin: 1:0.7 (a), 1:1.4 (b), 1:2.8 (c), and 1:5.5 (d).

stoichiometric, i.e. *n* equals 0.7, 1.4 and 2.8, respectively. However, when reaching a molar ratio of 1:5.5 (HSA:I) a sharp indomethacin zone appears, indicating that the albumin is saturated with bound indomethacin (fig.3d). By measuring the width of the indomethacin zone, the molar amount can be calculated from a pre-made calibration curve. In this case, the zone-width corresponds to 2.2 nmol indomethacin. Since the total amount of indomethacin added was 7.2 nmol (table 1), the amount of bound indomethacin is 5.0 nmol. The saturation ratio can now be calculated to be 1.3:5.0 which equals 1:3.9, indicating the binding of four indomethacin molecules to each molecule of albumin. However, the curved UV profile of the saturated albumin complex (fig.3d) may indicate a lower binding strength for the fourth indomethacin.

It is concluded that analytical isotachophoresis in a capillary tube, due to the very high resolution of the technique in combination with the possibility of quantitation, is a powerful tool for studying strong complex-formation systems, such as the protein–drug interaction studied in this work. Also in protein–protein interaction systems, it is possible to separate the complex from the constituent protein components. This was indicated by our preliminary studies on tryptic fragments of the staphylococcal protein A in its interaction with the Fc-fragment of immunoglobulin G. Furthermore, by thorough theoretical considerations of the shape of the UV-profile representing the complex, it should also be possible to study weakly-interacting systems, in which the complexes are disrupted during the separation. Such kinetically labile complex equilibria have been used in capillary isotachophoresis to increase the separating power of this technique [6].

Acknowledgements

The authors wish to express their thanks to Professor J. Sjöquist and to Dr R. Lindmark for stimulating discussions.

References

[1] Haglund, H. (1970) Science Tools 17, 1, 2–12.
[2] Arliager, L. (1974) J. Chromatogr. 91, 785–794.
[3] Everaerts, F. M., Beckers, J. L. and Verheggen, Th. P. E. M. (1976) Isotachophoresis, Theory, Instrumentation and Applications. Journal of Chromatography Library, vol. 6, Elsevier, Amsterdam.
[4] Hvidberg, E., Lausen, H. H. and Jansen, J. A. (1972) Eur. J. Clin. Pharmacol. 4, 119–124.
[5] Hultmark, D., Borg, K. G., Elofsson, R. and Palmer, L. (1975) Acta Pharm. Suec. 12, 259–276.
[6] Kaniansky, D. and Everaerts, F. M. (1978) J. Chromatogr. 148, 441–446.