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Measurement of flurbiprofen–human serum albumin interaction by fluorimetry

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Abstract: The binding of the anti-inflammatory drug, flurbiprofen, to human serum albumin is accompanied by a reduction of the fluorescence efficiency of the drug. The quenching effect has been used to evaluate the strength of binding at different pH values. Equations have also been developed for calculating binding constants for a 1:1 drug–protein complex, when fluorescence measurements are made at wavelengths where the emission spectra of the free drug, free protein and the complex overlap. Over the pH range 6.20–8.04, a binding constant of *ca* 1.0×10^7 was found for flurbiprofen.

Keywords: *Flurbiprofen; anti-inflammatory drug; human serum albumin; drug–protein association constants; fluorescence quenching.*

Introduction

Fluorimetric methods for the determination of the stability constants of drug–protein association complexes are among the simplest and most sensitive available [1–6]. *In situ* fluorimetry often obviates the necessity for laborious and time-consuming separations of free and bound drug fractions and allows greater freedom in selecting solution conditions, as high salt concentrations are not needed to overcome the Donnan equilibria associated with separations based upon semipermeable membranes.

The *in situ* fluorimetric determination of drug–protein association constants exploits the differences between the fluorescence efficiency of the free drug, the free protein and of the complex or complexes formed. The latter is sometimes referred to as the fluorescence efficiency of the bound drug. When possible, spectroscopic conditions are selected such that only the free drug or the free and the bound drug are excited. The emission monitored may be that of the free or bound drug. Alternatively, in the case of non-fluorescent drugs, the quenching of the fluorescence of the free protein, resulting from binding, may be monitored to obtain the quantitative data necessary to evaluate the binding constants.

In the present work, a situation is examined which is common, but rarely confronted, in the fluorimetric evaluation of binding equilibria. This is the case where not only do the

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free drug, the protein and the bound drug all fluoresce in the same spectral region, but also their excitation (or absorption) spectra overlap. Thus all three components must perforce be excited simultaneously, while their overlapping emission spectra lead to mixed fluorescence signals for quantitative analysis.

As an example, the formation of the 1:1 complex of the non-steroidal anti-inflammatory drug, flurbiprofen, with human serum albumin has been investigated. The method developed is useful for the case discussed above for overlapping excitation and emission spectra, when only a 1:1 complex is involved, a frequently occurring circumstance [5]; it should also be useful for circumstances where high ratio complexes are formed, in which the molar absorptivities and fluorescence efficiencies of the bound ligands in each kind of complex are identical, a surprisingly common occurrence [1].

Materials and Methods

Fluorimetric measurements were carried out using silica fluorescence cells (1 × 1 cm) and a Perkin–Elmer (Norwalk, CT, USA) model MPF-44A fluorescence spectrophotometer, operated in the energy mode, equipped with a Perkin–Elmer model 056 recorder. For the titration procedures, the excitation wavelength was 249 nm with emission observed at 310 nm, both slit widths being at 10 nm. Absorption spectrophotometric measurements were made using a Cary 219 spectrophotometer and 1-cm silica cells. A Beckman digital pH meter (model 4500) was used in the adjustment of pH values.

Human serum albumin (HSA) Fraction V, nitrogen content 15.9% (Lot No. 100F-02061) was obtained from Sigma Chemical Co. (St Louis, MO, USA). Sodium flurbiprofen dihydrate (FBP) (Batch No. 00314DY) was obtained from Boots Co. Ltd. (Nottingham, UK). Dowex 50 W-X58 (20–50 mesh) cation exchange resin and Dowex 1-X8 (20–50 mesh) anion exchange resin were obtained from J.T. Baker Chemicals (Phillipsburg, NJ, USA). Other reagents were of analytical reagent grade and were checked before use for the absence of fluorescence contaminants.

Deionized water was used in the preparation of all solutions. Albumin was deionized [7] and its concentration in solution was determined spectrophotometrically, taking its molecular weight as 66 500 and its molar absorptivity (ϵ) at 278 nm as 37 300 l $mole^{-1}$ cm^{-1}, following gravimetric analysis of the above batch. FBP solutions and HSA dilutions were prepared in 0.1 M phosphate buffer (pH 6 to 9) and 0.1 M borate buffer (pH 9 only).

Fluorimetric titrations were carried out by titrating 2.0 ml solution in a fluorimetric cell with successive aliquots of titrant, added by means of a Socorex Positive Displacement Micropipette model 841 (1–5 μl) (Alltech Associates, Deerfield, Illinois, USA).

FBP solutions (4×10^{-7}M) were titrated with HSA solutions (1×10^{-4}M). Similarly HSA solutions (5×10^{-7} to 8×10^{-7}M) were titrated with FBP solutions (1×10^{-4}M). Control titrations of the appropriate buffer solutions were also carried out in absence of drug or protein, using HSA and FBP solutions as titrants.

Results and Discussion

The absorption spectrum of flurbiprofen (FBP) (Fig. 1) in aqueous phosphate buffer (pH 7.40) shows a maximum at 246 nm and a shoulder at 275 nm; these features are overlapped by the HSA absorption spectrum. When excited at 249 nm, the FBP

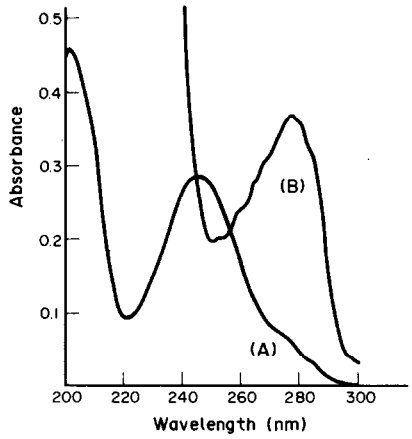

Figure 1
Ultraviolet absorption spectra at pH 7.40 in 0.1 M phosphate buffer: (A) FBP 1.49×10^{-5}M; and (B) HSA 9.92×10^{-6}M.

emission maximum is at 310 nm. This band is likewise overlapped by the emission band of HSA, although the intensity of interference from the albumin is reduced by exciting at its absorption minimum (249 nm). When FBP binds to HSA, the fluorescence spectrum of the drug is quenched, while that of the albumin shows little or no change (Fig. 2).

Titration of FBP with HSA

Determinations were carried out in phosphate buffers at pH values of 6.20, 6.80, 7.40 and 8.04, respectively, and in borate and phosphate buffers at pH 9.04, as illustrated in the typical curves shown in Fig. 3. FBP fluorescence decreases sharply during titration with HSA, reaching a minimum corresponding to a protein:drug (P/D) ratio of about 2. After this as titration continues, the fluorescence gradually increases until the curve runs parallel with the control curve, when the fluorescence observed is due to the fully-bound drug plus excess unbound HSA, the drug–protein complex having a slightly higher fluorescence intensity than the free protein. Control titration curves were identical at the different pH values, and remained linear up to a P/D ratio corresponding to at least 10. The final part of curve C at pH 9.04 (Fig. 3) is exceptional, in that it does not run parallel to the control curve, irrespective of whether phosphate or borate buffer is used. Other titration curves were similar to curve A at pH 7.40, although at pH 8.04 the final slope was closer to the control slope; at pH 9.04, the initial slope of the titration curve was less well defined.

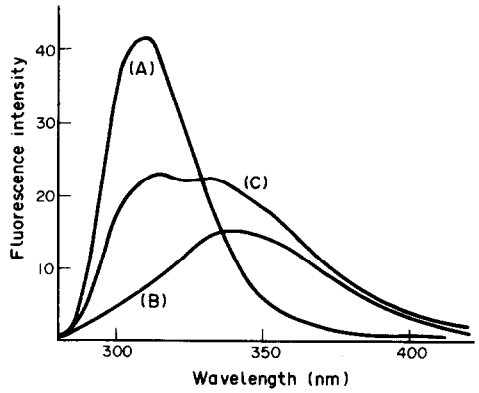

Figure 2
Emission spectra obtained at pH 7.40 in 0.1 M phosphate buffer containing: (A) FBP 2.05×10^{-6}M; (B) HSA 2.73×10^{-6}M; and (C) a mixture of FBP 1.86×10^{-6}M and HSA 2.73×10^{-6}M. Excitation wavelength, 249 nm; spectral bandwidth, 10 nm.

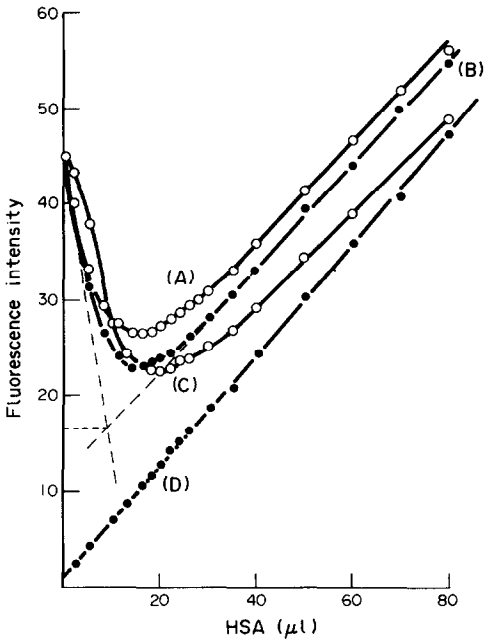

Figure 3
Fluorescence quenching curves of FBP 4.09 × 10^{-7}M (2.0 ml) titrated by HSA: (A) 1.02 × 10^{-4}M at pH 7.40; (B) 1.04 × 10^{-4}M at pH 8.04; (C) 0.96 × 10^{-4}M at pH 9.04 (borate buffer); and (D) control titration curve of pH 7.40 buffer with addition of HSA 1.02 × 10^{-4}M.

Binding constants were calculated by two methods. The first method is applicable only under conditions where there is no background protein fluorescence (as in a previous study of phenprocoumon–albumin binding; [3]), or where the fluorescence due to protein is invariant on binding. Drug–HSA fluorescence measurements, obtained during titration, were corrected by subtracting the corresponding control fluorescence measurements, obtained by addition of HSA alone. Corrected fluorescence (CF) intensity values so obtained were plotted as a function of HSA concentration (Fig. 4), producing a smooth exponential curve which, with the addition of excess HSA, reached a lower limit corresponding to complete binding of the drug.

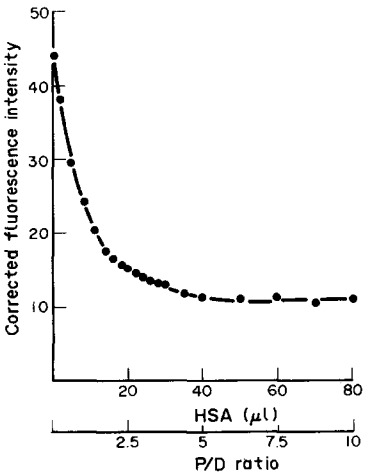

Figure 4
Corrected fluorescence quenching curve for FBP 4.09 × 10^{-7}M (2.0 ml) with addition of HSA 1.02 × 10^{-4}M at pH 7.40, showing the protein:drug (P/D) ratio.

Where a 1:1 drug–protein complex is formed, the fraction of drug bound (X_D) at a given intermediate concentration of HSA can be calculated using the expression:

$$X_D = \frac{CF_{max} - CF}{CF_{max} - CF_{min}}$$

where CF_{max} and CF_{min} are the upper and lower values of corrected fluorescence intensity corresponding to unbound and fully-bound drug respectively, while CF is the value obtained at the intermediate HSA concentration specified.

Analysis of the corrected titration curve data by an iterative least squares technique (SAS 82 PROC NLIN) was accordingly carried out to determine the binding constant (K) at different pH values.

To ascertain the validity of this approach for this particular determination, an alternative method of calculation based on the original curve data was also used, as described below.

During the fluorimetric titration, before the drug becomes fully bound, the total fluorescence (F) at emission wavelength λ_f is:

$$F = F_P + F_{PD} + F_D$$

On substituting the usual equation for fluorescence intensity this becomes:

$$F = 2.303 I_o d\{\phi_P \epsilon_P [P] + \phi_{PD} \epsilon_{PD} [PD] + \phi_D \epsilon_D [D]\}$$

where [P], [PD] and [D] are the molar concentrations and F_P, F_{PD} and F_D are the corresponding fluorescence intensities due to free protein, bound drug and free drug, respectively; I_o is the intensity of the incident excitation radiation at λ_{ex}; ϵ is the molar absorptivity; ϕ is the quantum yield; and d is the solution path length in cm.

If F_P^o is the fluorescence intensity of added protein (control curve), F_{PD}^o is the fluorescence of the fully-bound drug, F_D^o is the fluorescence intensity of the free drug before addition of protein, and C_P and C_D are the molar concentrations of protein and drug added at a given point in the titration curve; then it can also be stated that:

$$F_P^o = 2.303 I_o \phi_P \epsilon_P C_P d \text{ and thus, } \frac{F_P^o}{C_P} = 2.303 I_o \phi_P \epsilon_P d.$$

$$\text{Similarly, } \frac{F_{PD}^o}{C_D} = 2.303 I_o \phi_{PD} \epsilon_{PD} d$$

$$\text{and } \frac{F_D^o}{C_D} = 2.303 I_o \phi_D \epsilon_D d.$$

Substitution in the equation for total fluorescence gives:

$$F = F_P^o \frac{[P]}{C_P} + F_{PD}^o \frac{[PD]}{C_D} + F_D^o \frac{[D]}{C_D}.$$

By further substitution in the equation above, the following expression can be derived for the concentration of free drug [D] since:

$$[PD] = C_D - [D]$$

$$\text{and } [P] = C_P - [PD] = C_P - C_D + [D].$$

$$[D] = \frac{C_D C_P (F - F^\circ_{PD} - F^\circ_P (1 - \frac{C_D}{C_P}))}{C_P (F^\circ_D - F^\circ_{PD}) + F^\circ_P C_D}.$$

Thus the binding constant, K, is given by:

$$K = \frac{[PD]}{[P][D]}.$$

F°_{PD} cannot be measured directly, but can be estimated by extrapolating the initial and final slopes of the titration curve as shown in Fig. 3, or preferably by calculation, since after the drug becomes fully bound:

$$F = F^\circ_P \frac{[P]}{C_P} + F^\circ_{PD} \frac{[PD]}{C_D}.$$

As all the drug is in the PD form,

$$[PD] = C_D \text{ and } \frac{[PD]}{C_D} = 1.$$

Hence, $F = F^\circ_P \frac{[P]}{C_P} + F^\circ_{PD}$

$$[P] = C_P - [PD] = C_P - C_D.$$

Therefore, $F^\circ_{PD} = F - F^\circ_P \frac{(C_P - C_D)}{C_P}$.

Taking a number of values of F and F°_P corresponding to the final slope of the titration curve, calculated values of F°_{PD} were in good agreement with extrapolated values, except at pH 9.04 where the extrapolated values were higher.

Values of K were calculated using data taken from at least seven points on the titration curve. The mean binding constants calculated by the two methods of evaluation are shown in Table 1. Results at pH 9.04 differ by up to 55% and are inconclusive, as expected from the final slope of the titration curve. Over the pH range 6.20–8.04, the K values calculated by the two methods are in somewhat better agreement, and they appear to be pH-independent.

Table 1
Binding constants determined by two calculation procedures from fluorescence quenching measurements in the titration of FBP 4.09×10^{-7}M (2.0 ml) with HSA

		Binding constant ($K \times 10^7$) From fluorescence equations		From corrected curves		
pH	HSA titrant ($M \times 10^{-4}$)	$K \times 10^7$	Standard deviation	$K \times 10^7$	Asymptotic standard error	Asymptotic 95% confidence interval
6.20	1.02	1.17	0.04	0.95	0.05	0.85–1.10
6.80	1.02	1.38	0.10	1.07	0.13	0.79–1.36
7.40	1.02	0.89	0.02	0.92	0.11	0.69–1.15
8.04	1.04	1.10	0.06	0.96	0.04	0.87–1.05
9.04*	0.96	0.57	0.15	0.40	0.05	0.29–0.52
9.04	1.04	0.42	0.10	0.24	0.03	0.17–0.30

* Using borate buffer. All other solutions employed phosphate buffer (see text).

Titration of HSA with FBP

Typical fluorimetric titration and control titration curves determined at pH 7.40 are shown in Fig. 5; similar curves were obtained at pH values of 6.20, 6.80, 8.04 and 9.04. All the titration curves show an initial sharp decrease in fluorescence intensity, after which they gradually diverge from, instead of running parallel to, the corresponding linear control curves up to a drug:protein (D/P) ratio of 6 or more. The extent of the divergence was less pronounced at pH 6.20 and 6.80 than at higher pH values.

The decrease in drug fluorescence produced on binding was calculated by subtracting the fluorimetric titration values from the corresponding control titration values, and then plotted as a function of HSA concentration (Figs 6 and 7). Calculation of the fraction of

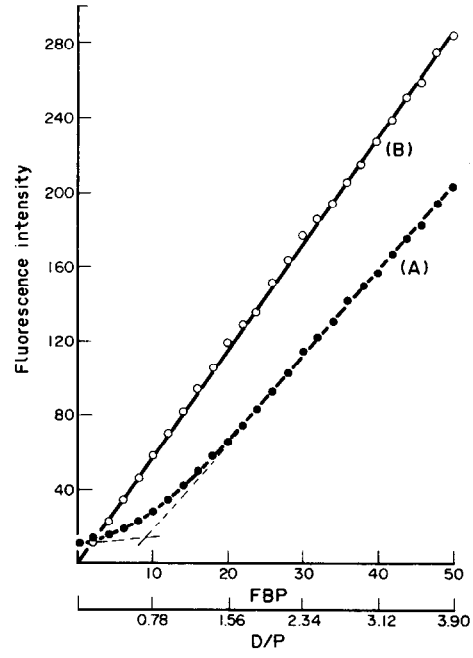

Figure 5
Fluorescence curves of (A) HSA 7.89×10^{-7}M (2.0 ml) and (B) 0.1 M phosphate buffer (2.0 ml) on addition of FBP 1.23×10^{-4}M, showing the drug: protein (D/P) ratio.

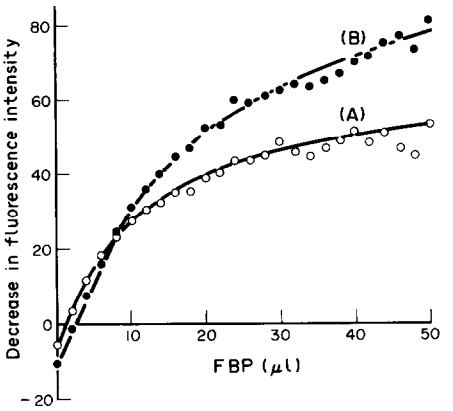

Figure 6
Fluorescence decrease produced by HSA (A) 4.93×10^{-7}M and (B) 7.89×10^{-7}M with addition of FBP 1.23×10^{-4}M at pH 7.40.

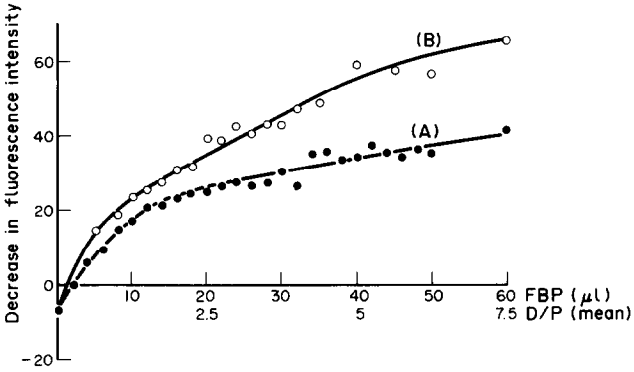

Figure 7
Fluorescence decrease produced by HSA (A) 5.00×10^{-7}M at pH 6.20 and (B) 4.80×10^{-7}M at pH 9.04 with addition of FBP 1.23×10^{-4}M.

protein bound (X_P) from these curves was not feasible, probably because binding continues to occur at lower-affinity sites. As a result, the analysis of the data by the iterative least squares technique gave K values which were much lower than those obtained when HSA was used as titrant.

In the alternative method of calculation from titration curve data, the expression for the fluorescence of bound drug becomes:

$$F^{\circ}_{PD} = 2.303 I_o \phi_{PD} \epsilon_{PD} C_P d.$$

The derived equation for calculating the concentration of free drug [D] thus becomes:

$$[D] = \frac{(C_D C_P (F - F^{\circ}_P + (F^{\circ}_P - F^{\circ}_{PD}) \frac{C_D}{C_P}))}{C_P F^{\circ}_D + C_D (F^{\circ}_P - F^{\circ}_{PD})}.$$

The expression for calculating F°_{PD} can be derived from the equation:

$$F = F^{\circ}_D \frac{[D]}{C_D} + F^{\circ}_{PD} \frac{[PD]}{C_P}.$$

As all the protein is in the PD form,

$$[PD] = C_P \text{ and } \frac{[PD]}{C_P} = 1.$$

Hence, $F^{o}_{PD} = F - F^{o}_{D} \dfrac{(C_D - C_P)}{C_D}$.

In this instance, however, since the final slope of the titration curve does not run parallel to the control slope, F^{o}_{PD} values could not be calculated. Approximate values of F^{o}_{PD} were obtained by extrapolation (Fig. 5), and used to calculate the K values shown in Table 2. Because of the uncertainty in determining F^{o}_{PD}, the binding constants are less reliable than those shown in Table 1, but the determinations are useful since they show that HSA concentration can be varied without altering the K values obtained.

Table 2
Binding constants determined from fluorescence quenching measurements in the titration of HSA (2.0 ml) with flurbiprofen 1.23×10^{-4}M. Anomalous results are obtained by calculation from difference curves

		Binding constant (K) From fluorescence equations		From difference curves		
pH	HSA ($M \times 10^{-7}$)	$K \times 10^{7}$	Standard deviation	$K \times 10^{7}$	Asymptotic standard error	Asymptotic 95% confidence interval
6.20	5.00	0.66	0.10	0.21	0.03	0.15–0.28
	6.00	0.57	0.11	0.75	0.14	0.47–1.03
	8.00	0.64	0.02	0.20	0.02	0.15–0.25
6.80	5.00	0.91	0.03	0.47	0.08	0.29–0.64
	6.00	0.97	0.12	0.27	0.05	0.17–0.37
	8.00	0.73	0.09	0.29	0.03	0.22–0.35
7.40	4.93	1.14	0.05	0.27	0.03	0.21–0.33
	5.92	1.09	0.05	0.33	0.05	0.24–0.43
	7.89	0.98	0.04	0.17	0.02	0.14–0.21
8.04	4.95	0.62	0.08	0.26	0.05	0.16–0.37
9.04*	4.80	0.72	0.08	0.08	0.01	0.05–0.10

* Using borate buffer. All other solutions employed phosphate buffer (see text).

The binding constants shown in Tables 1 and 2 are several times lower than those obtained by equilibrium dialysis [8, 9]. However, in the dialysis method protein concentrations higher by two orders of magnitude were used. This concentration effect is frequently noticed [10, 11] and is probably attributable to the increased protein–protein interaction at higher concentrations, which leads to a decrease in the availability of binding sites. Surprisingly, these fluorescence data do not indicate that the binding of flurbiprofen to human serum albumin is pH-dependent, as does the dialysis method [8]. In previous studies [3, 4, 12] with coumarins, the fluorescence method has readily detected changes in K over the pH range 5–9; these changes are associated with the N–B conformational change of albumin [12–14].

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References

[1] D. V. Naik, W. L. Paul, R. M. Threatte and S. G. Schulman, *Anal. Chem.* **47**, 267–270 (1975).
[2] D. V. Naik, W. L. Paul and S. G. Schulman, *J. Pharm. Sci.* **64**, 1677–1680 (1975).
[3] M. Otagiri, J. S. Fleitman and J. H. Perrin, *J. Pharm. Pharmacol.* **32**, 478–482 (1980).
[4] M. Otagiri, Y. Otagiri and J. H. Perrin, *Int. J. Pharm.* **2**, 283–294 (1979).
[5] M. R. Loken, J. W. Hayes, J. R. Gohlke and L. Brand, *Biochemistry* **11**, 4779–4785 (1982).
[6] A. El-Nimr, G. E. Hardee and J. H. Perrin, *J. Pharm. Pharmacol.* **33**, 117–118 (1981).
[7] J. S. Fleitman and J. H. Perrin, *Int. J. Pharm.* **11**, 215–225 (1982).
[8] H. Ueda and J. H. Perrin, to be published.
[9] P. C. Risdall, S. S. Adams, E. L. Crampton and B. Marchant, *Xenobiotica* **8**, 691–704 (1978).
[10] C. J. Bowmer and W. E. Lindup, *J. Pharm. Sci.* **67**, 1193–1195 (1978).
[11] C. J. Bowmer and W. E. Lindup, *Biochem. Pharmacol.* **27**, 937–942 (1978).
[12] J. Wilting, W. F. van der Giesen, L. H. M. Janssen, M. M. Weideman, M. Otagiri and J. H. Perrin, *J. Biol. Chem.* **255**, 3032–3037 (1980).
[13] J. Wilting, M. M. Weideman, A. C. J. Roomer and J. H. Perrin, *Biochim. Biophys. Acta* **579**, 469–473 (1979).
[14] J. S. Fleitman and J. H. Perrin, *Int. J. Pharm.* **11**, 227–236 (1982).

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