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STUDY OF DEUTERO-ISOTOPOMER-INDUCED INHIBITION OF CAFFEINE AND PHENOBARBITONE BINDING TO HUMAN SERUM ALBUMIN

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Abstract—The present study of inhibition provides confirmation to previously observed deuterium isotope effects on *in vitro* caffeine and phenobarbitone binding to human serum albumin (HSA). Addition of either 3,7($C(^2H)_3)_2$ or 1,3,7($C(^2H)_3)_3$ caffeine induces a 50% loss in both the extent of binding and binding parameters of the unlabelled analog, understandably so in view of the stronger individual HSA binding of the two labelled isotopomers.

As concerns caffeine displacement from its HSA sites, we show phenobarbitone and its 5-pentadeuterophenyl analog are equally potent inhibitors of caffeine binding, though their individual HSA binding profiles differ.

As for HSA binding interactions between phenobarbitone isotopomers, a 50% decrease in unlabelled phenobarbitone extent of binding is observed in the presence of its 5-pentadeuterophenyl analog. Our results favor the hypothesis of differing binding sites for each isotopomer.

In the course of treatments associating two or more drugs, bound ligands may be displaced from their plasma protein binding sites (HSA sites here), the intensity of this phenomenon depending both upon the nature of the binding site, protein concentration and relative concentrations of the competing substances together with the physico-chemical properties of the ligand molecules.

Protein binding inhibition may result from competitive or non-competitive interaction between ligands depending on whether these have identical binding sites on the protein or not [1–3].

Optimal situation for the generation of binding interactions is encountered with molecules bound to a limited number of HSA sites according to a saturable binding mode, so that binding saturation is reached within the therapeutic blood concentrations range [4–6].

Since caffeine [7] and phenobarbitone [8] HSA bindings are of the saturable mode, the search for HSA binding isotope effects for these two molecules together with the study of eventually associated labelled isotopomer-induced inhibition of unlabelled analog binding may provide a fruitful approach from several standpoints.

(i) Isotope effects may affect both partition coefficients [9–11] and hydrogen bonds [12, 13], while hydrophobic and hydrogen bondings are the two major factors accounting for drug protein binding [14, 15] and isotope effects have already been reported for phenytoin binding [16].

(ii) In the majority of protein binding studies performed by equilibrium dialysis, drug quantitation in each compartment of the dialysis cell resorts to radioactive isotope labelling techniques which, together with the greater capacity of radioactive labels to induce isotope effects relatively to stable isotopes (larger mass difference from the major isotope), points to the necessity to evaluate the involvement of various molecular sites in the binding process so as to design tracer isotopomers devoid of isotope effects. In the process, useful mechanistic information may be derived concerning the drug's fine mode of action.

(iii) In the precise case of caffeine [17] and phenobarbitone, multisite labelling is accompanied by differential alterations in HSA binding parameters.

The present study of protein binding inhibition is intended to confirm the latter isotope effects and show that these effects may stem from altered physico-chemical properties of the labelled analogs.

MATERIALS AND METHODS

Reagents and isotopomers. Structures of caffeine and phenobarbitone isotopomers are presented in Fig. 1.

Unlabelled caffeine (1,3,7-trimethyl xanthine) and phenobarbitone (5-ethyl,5-phenyl barbituric acid) as well as A 1887 fatty acid-free human serum albumin were purchased from Sigma Chemicals (St Louis, MO).

3,7($C(^2H)_3)_2$- and 1,3,7($C(^2H)_3)_3$ caffeine were synthesized as described previously [18].

1,3-^{15}N;2-^{13}C caffeine and phenobarbitone internal standards (isotopic enrichment: $^{13}C > 90\%$, $^{15}N > 99\%$) as well as 5(phenyl$(^2H)_5$)-phenobarbitone (isotopic enrichment > 99%) were obtained from CEA (Commissariat à l'Energie Atomique, Saclay, France).

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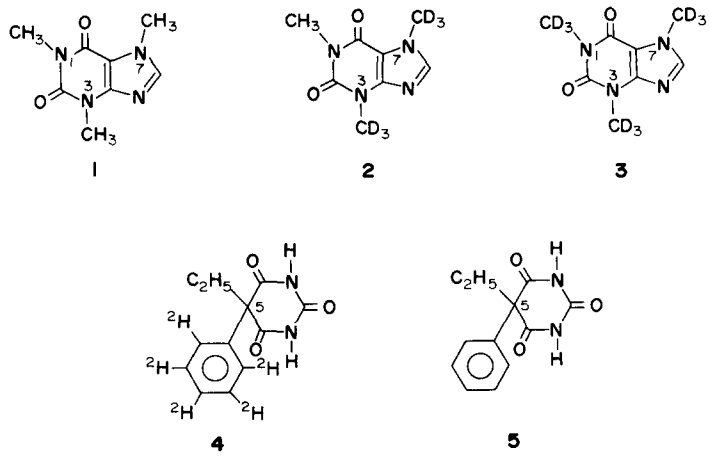

Fig. 1. Structure of caffeine and phenobarbitone isotopomers: (1) unlabelled caffeine, (2) 3,7($C[^2H]_3)_2$-caffeine, (3) 1,3,7($C[^2H]_3)_3$-caffeine, (4) 5(phenyl$[^2H]_5$)-phenobarbitone, (5) unlabelled phenobarbitone.

All solvents and reagents used were analytical grade.

Protein binding studies. HSA binding studies were performed by equilibrium dialysis in a DIANORM® apparatus bearing 20 cells (0.2 × 0.2 ml), this at pH = 7.4 (Sorensen's phosphate buffer) and 37° for 3 hr.

Concentration ranges used are identical to those previously employed for the study of caffeine [17] and phenobarbitone individual bindings: 50–10,000 μM for caffeine, 25–10,000 μM for phenobarbitone.

Concentrations of the added binding inhibitor molecules are fixed and of the order of their dissociation constants (K_d) i.e. 3000 μM (final concentration) for 3,7($C(^2H)_3)_2$-caffeine, 2000 μM (final concentration) for 1,3,7($C(^2H)_3)_3$ caffeine and 5(phenyl$(^2H)_5$)-phenobarbitone.

Following equilibrium dialysis, free (F) and bound (B) caffeine and phenobarbitone were quantitated by gas chromatography coupled to mass selective detection (GC-MS) after the method of Désage [19] and Benchekroun [20] using internal standards 1,3-^{15}N;2-^{13}C-caffeine and phenobarbitone for the respective quantitation of caffeine and phenobarbitone analogs.

Calculation of drug HSA binding parameters. Measurement of B and F permits calculation of HSA binding parameters N, n, K_a and P after the B vs F plot according to:

$$B = N \cdot K_a \cdot F/(1 + K_a \cdot F) = n \cdot K_a \cdot F/(1 + K_a \cdot F)$$

where P is the total HSA concentration, K_a the drug protein association constant or affinity constant, N the total sites concentration, n the average number of binding sites per HSA molecule, and nP the total concentration of drug binding sites. From B and F values, it is also possible to calculate the extent of drug binding and draw the B/F vs B plot (Scatchard plot) as:

$$B/F = -BK_a + NK_a$$

B vs F and Scatchard plot-drawings as well as calculation of binding parameters used program "Triomphe" [21] serving on a 4052 Tektronix calculator.

Statistical treatment of HSA binding data. Student's t-test and Fischer's F-test were used to compare unlabelled drug binding in the presence of each inhibitory isotopomer.

RESULTS

Each data point used for calculation of HSA binding parameters (given as mean ± SD) is the mean of two measurements.

Inhibition of caffeine HSA binding by 3,7-ditrideuteromethyl caffeine

In the presence of 3000 μM 3,7($C(^2H)_3)_2$-caffeine, the extent of unlabelled caffeine binding to serumalbumin (binding is of the saturable type) is reduced from 13% to 4% on rising (50–8000 μM) unlabelled caffeine concentration (Fig. 2).

Caffeine binding parameters determined in the same conditions after the Scatchard plot are (Fig. 2): $K_a = 268 \pm 40\ M^{-1}$ (vs $486 \pm 35\ M^{-1}$ in the absence of binding inhibitor), $N = 444 \pm 31$ μM (vs 822 ± 80 μM) and $n = 0.74$ (vs 1.00).

Inhibition of caffeine binding in the presence of 1,3,7-tri-trideuteromethyl caffeine

When unlabelled caffeine equilibrium dialysis is performed in the presence of 2000 μM 1,3,7-($C(^2H)_3)_3$-caffeine, the bound drug form falls from 13% to 4% on increasing (50–9000 μM) concentrations of the unlabelled molecule (Fig. 3). Caffeine HSA binding parameters observed here are (Fig. 3): $K_a = 341 \pm 109\ M^{-1}$, $N = 535 \pm 69$ μM and $n = 0.89$.

Inhibition of unlabelled caffeine HSA binding by two phenobarbitone isotopomers

In a former study we have shown the extents of phenobarbitone and its 5-pentadeuterophenyl isotopomer HSA binding differ.

It may be that the capacity of phenobarbitone to

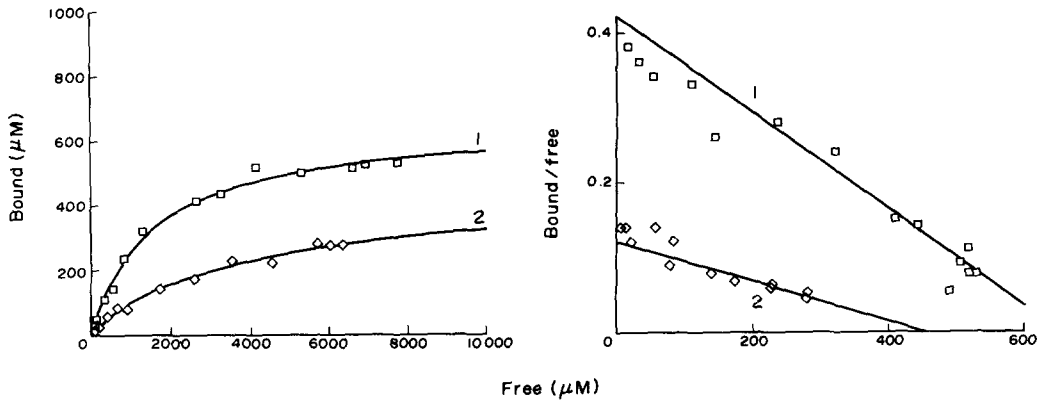

Fig. 2. Caffeine HSA binding: (1) unlabelled caffeine alone; (2) in the presence of 3,7($C[^2H]_3$)$_2$-caffeine. Each data point corresponds to the mean of two measurements. SEM for each data point = 5% (comprised in symbols).

inhibit protein binding of other drugs (in the present case caffeine) is also affected by deuterium labelling on the aromatic ring, which we intended to investigate here.

In the presence of 2000 μM unlabelled phenobarbitone, caffeine extent of HSA binding decreases from 21% to 5% as its concentration rises in the range 50–10,000 μM (Fig. 4). Calculated caffeine binding parameters are: $K_a = 369 \pm 30\ M^{-1}$ (vs $486 \pm 35\ M^{-1}$ for caffeine alone) and $N = 600 \pm 27$ μM (vs 822 ± 80 μM).

Comparable figures are observed for caffeine HSA binding inhibition in the presence of 2000 μM 5-(phenyl(^2H)$_5$)-phenobarbitone, with a reduction in bound fraction from 19% to 4% on rising (50–10,000 μM) caffeine concentrations. Affinity constant and total number of HSA sites are $258 \pm 60\ M^{-1}$ and 517 ± 25 μM respectively (Fig. 4a and c).

Inhibition of phenobarbitone binding in the presence of its 5-pentadeuterophenyl isotopomer

5-(Phenyl(^2H)$_5$)-phenobarbitone, which has higher HSA affinity than its unlabelled counterpart ($K_a = 942 \pm 90\ M^{-1}$ vs $524 \pm 46\ M^{-1}$) is capable of inhibiting binding of the latter when added (2000 μM) to phenobarbitone dialysis medium, the same way as deutero-isotopomers inhibit caffeine binding: under such conditions unlabelled phenobarbitone bound fraction ranges from 33% to 8% as its concentration increases from 25 to 10,000 μM (Fig. 5), whereas usual binding parameters are reduced to $K_a = 275 \pm 56\ M^{-1}$ (down from $524 \pm 46\ M^{-1}$ for phenobarbitone alone) and $N = 1158 \pm 124$ μM (vs 1874 ± 77 μM).

DISCUSSION

The above presented results provide confirmation of the previously observed isotope effects on caffeine isotopomers individual binding [17] namely that extent of binding increases on successive trideuteromethyl group incorporation (42 and 46% for 3,7($C(^2H)_3$)$_2$-caffeine and 1,3,7($C(^2H)_3$)$_3$-caffeine respectively vs 27% for the unlabelled drug). Effectively the addition of 3,7 di- or 1,3,7 tri-trideuteromethyl caffeine in the course of caffeine dialysis reduces the fraction of drug bound by *ca.* 50% (the latter is 13% in both cases down from

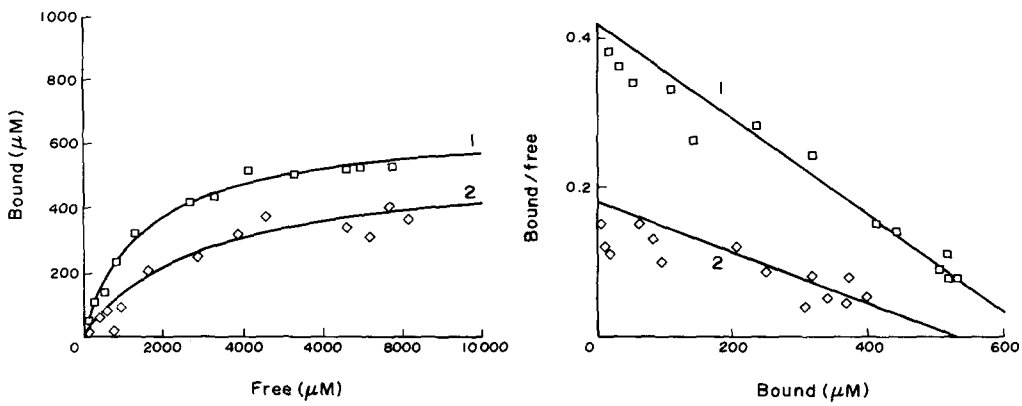

Fig. 3. Caffeine HSA binding: (1) unlabelled caffeine alone; (2) in the presence of 1,3,7($C[^2H]_3$)$_3$-caffeine. Each data point corresponds to the mean of two measurements. SEM for each data point = 5% (comprised in symbols).

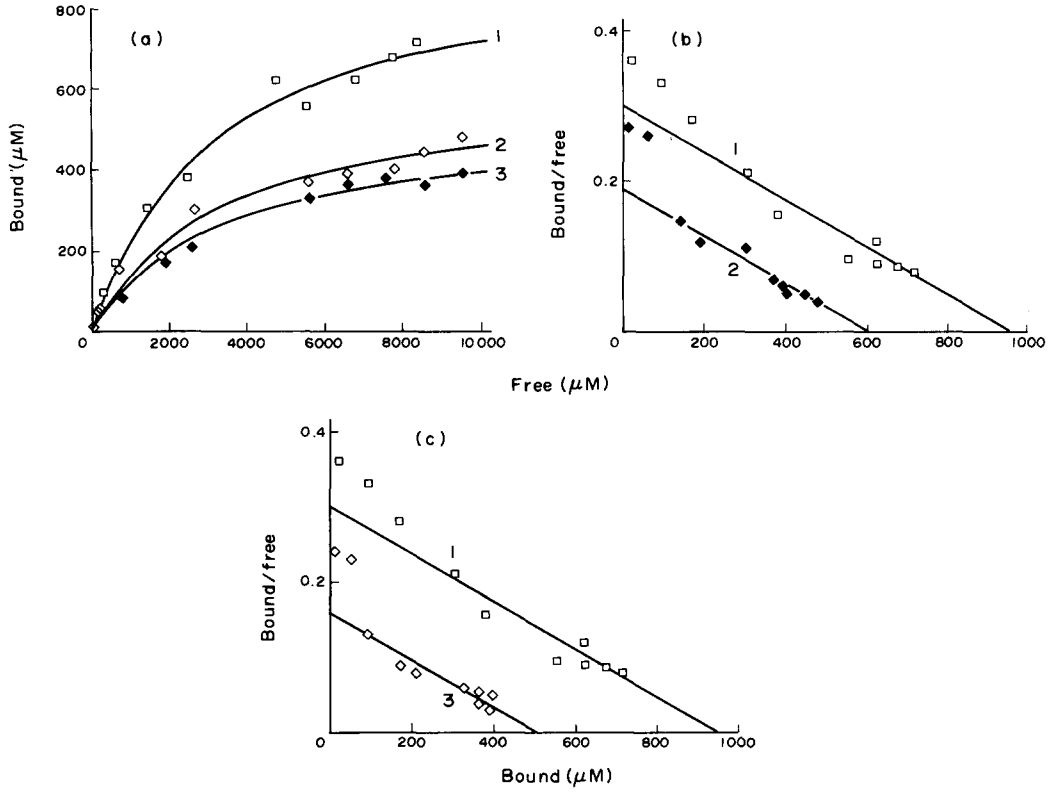

Fig. 4. Caffeine HSA binding: (1) unlabelled caffeine alone; (2) in the presence of unlabelled phenobarbitone; (3) in the presence of 5-(phenyl[^{2}H]$_5$)-phenobarbitone. Each data point corresponds to the mean of two measurements. SEM for each data point = 5% (comprised in symbols).

27% for inhibitor-free dialysis, this at 50 μM caffeine concentration). Total sites concentration N, is also significantly reduced with respective isotope effects N_H/N_{2_H} = 0.54 and 0.65 for 3,7 $(C(^2H)_3)_2$-caffeine and 1,3,7$(C(^2H)_3)_3$-caffeine inhibition; (H and ^{2}H stand for unlabelled and the deutero analog of interest).

However, 3,7 di-trideuteromethyl caffeine is a more potent caffeine inhibitor than 1,3,7 trideuteromethyl caffeine: on rising caffeine total concentration (50–8000 μM), caffeine bound concentrations range between 6 and 277 μM in the first case vs 6–367 μM when 1,3,7$(C(^2H)_3)_3$-caffeine is used as HSA binding inhibitor.

Other caffeine HSA binding parameters are also more affected by addition of the hexadeutero analog (K_a = 268 ± 40 M^{-1} vs 341 ± 109 M^{-1}, N = 444 ± 31 μM vs 535 ± 69 μM, n = 0.74 vs 0.89).

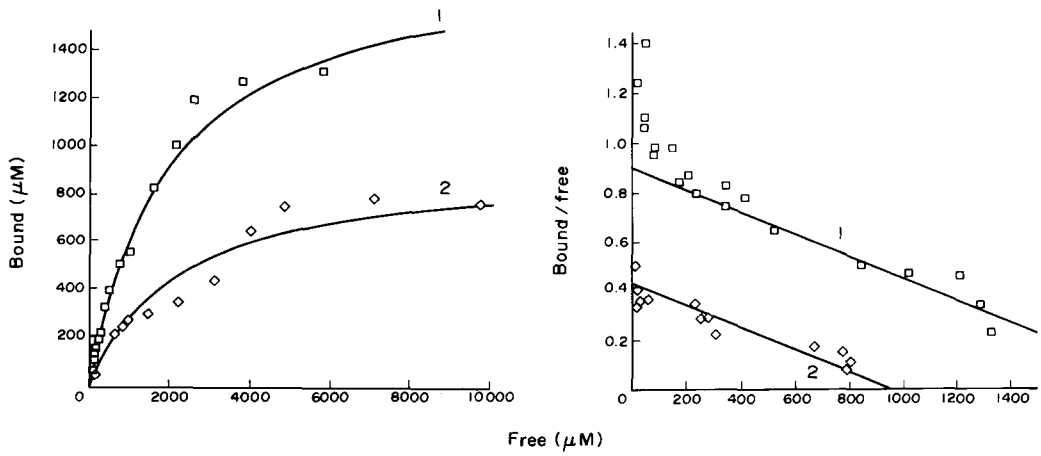

Fig. 5. Phenobarbitone HSA binding: (1) unlabelled phenobarbitone alone; (2) in the presence of 5-(phenyl[^{2}H]$_5$)-phenobarbitone. Each data point corresponds to the mean of two measurements. SEM for each data point = 5% (comprised in symbols).

As for determining the type of caffeine binding inhibition, the feeble HSA binding of both unlabelled and the two inhibitory trideuteromethyl isotopomers does not allow us to distinguish between the competitive and non-competitive mode, though the choice of concentrations of inhibitor isotopomers as their K_d is considered by the majority of the authors the most discriminative in such instances. The absence of a significant difference between K_a for isolated caffeine dialysis and dialysis in the presence of the 1,3,7($C(^2H)_3$)$_3$-isotopomer suggests HSA binding inhibition is of the non-competitive type in this case, though definitive evidence is still needed concerning this point: effectively the fact that caffeine and its isotopomers are only weakly bound to HSA (high K_d) requires use of large inhibitor concentrations, which precludes the benefits expectable from the method.

The above described deuteromethyl isotopomer-mediated caffeine HSA binding inhibition phenomena are attributable, partly at least, to physicochemical isotope effects, through the production, for example, of differential changes in the environment of HSA sites on going from unlabelled to deuterocaffeine binding. Such changes may stem from alterations in the strength and/or range of drug–protein hydrogen and hydrophobic bonds on N3 and N7 methyl proton deuteration [14, 15], since we have shown these two caffeine positions are strongly involved in caffeine HSA binding. This, we think, further ^{1}H-NMR relaxation studies will help define.

Another observation pointing to the importance of physicochemical parameters in isotopomer-induced binding inhibition phenomena is represented by a reduction in k' (HPLC capacity factor) on injecting pure solutions of the three caffeine isotopomers on one side and labelled–unlabelled caffeine mixture solutions on the other (Hewlett-Packard C18-ODS Hypersil 5 μm 200 × 4.6 mm column, mobile phase: THF/H_2O (0.5/250)). The average measured k' difference in this chromatographic system for the mixture caffeine–3,7($C(^2H)_3$)$_2$–caffeine is 0.6 down from 1.5 for separate isotopomer-solution injections, while respective figures for the couple caffeine–1,3,7($C(^2H)_3$)$_3$–caffeine are 1.2 and 3.0.

Considering the differing HSA binding parameters we had previously observed for 5(phenyl($^2H)_5$) phenobarbitone ($K_a = 942 \pm 90\ M^{-1}$; $N = 1088 \pm 42\ \mu M$; $n = 2$; bound fraction = 46%) vs $K_a = 524 \pm 46\ M^{-1}$; $N = 1874 \pm 77\ \mu M$; $n = 3$; bound fraction = 58% for the unlabelled analog, the observation of inhibition phenomena of the non-competitive type (K_a for phenobarbitone is significantly altered in the presence of its deuterophenyl counterpart) suggests each of the two isotopomers is bound to different serum albumin sites, though this interpretation needs be further confirmed.

As to the question whether unlabelled and 5(phenyl ($^2H)_5$) phenobarbitone are equally potent inhibitors of caffeine binding to HSA, our results indicate similar non-competitive inhibition (N is significantly reduced) upon addition of either phenobarbitone analog, in the course of the dialysis process, suggesting caffeine and the two phenobarbitone isotopomers are bound to distinct HSA sites. Here too, our approach yields only partial evidence, however, and we think a comparative dialysis study using concentrations of binding inhibitors different from their K_d may constitute a valuable confirmatory tool to our hypothesis.

In summary, the present HSA binding inhibition trials provide confirmation of isotope effects previously observed on caffeine and phenobarbitone individual HSA binding, namely that: (i) the extent of caffeine binding increases upon trideuteromethylation at molecular positions N3 and N7; (ii) conversely to 5-pentadeuteroethyl substitution, introduction of five phenyl-ring deuterium atoms reduces phenobarbitone HSA binding extent and alters the drug's nominal number of HSA binding sites.

Moreover our results suggest different sites are implicated in caffeine and phenobarbitone HSA binding processes.

REFERENCES

1. R. Zini, State Thesis, Paris XII (1984).
2. E. M. Sellers, in *Advances in Pharmacology and Therapeutics*, Vol. 7 (Ed. J. P. Tillement), p. 153. Pergamon Press, Oxford (1980).
3. U. Kragh-Hansen, *Pharmac. Rev.* **33**, 17 (1981).
4. L. J. Aarons, W. L. Schary and M. Rowland, *J. Pharm. Pharmac.* **31**, 322 (1979).
5. A. Kober, Y. Olsson and I. Sjoholm, *Molec. Pharmac.* **18**, 237 (1980).
6. I. Odar-Cedereof and O. Borga, *Clin. Pharmac. Ther.* **20**, 36 (1976).
7. E. Zylber-Katz, L. Granit and M. Levy, *Clin. Pharmac. Ther.* **36**, 133 (1984).
8. W. J. Waddell and T. C. Butler, *J. clin. Invest.* **36**, 1217 (1957).
9. N. El Tayar, H. Van de Waterbeemd, M. Gryllaki, B. Testa and W. F. Trager, *Int. J. Pharm.* **19**, 271 (1984).
10. N. Tanaka and E. R. Thornton, *J. Am. chem. Soc.* **98**, 1617 (1976).
11. M. G. Horning, J. P. Thenot, O. Bouswsma, J. Nowlin and K. Lertratanangkoon, in *Advances in Pharmacology and Therapeutics*, Vol. 7 (Ed. J. P. Tillement), p. 245. Pergamon Press, Oxford (1980).
12. K. I. Tomita, A. Rich, C. De Loze and E. R. Blout, *J. molec. Biol.* **4**, 83 (1962).
13. H. A. Scheraga, *Ann. N.Y. Acad. Sci.* **84**, 608 (1960).
14. M. Le Maire and J. P. Tillement, *Biochem. Pharmac.* **31**, 359 (1982).
15. S. Urien, E. Albengres, R. Zini and J. P. Tillement, *Biochem. Pharmac.* **31**, 3687 (1982).
16. J. P. Thenot, T. I. Ruo, P. Stec and A. J. Atkinson, Jr., in *Recent Developments in Mass Spectrometry in Biochemistry and Medicine*, Vol. 6 (Eds. A. Frigerio and M. McCamish), p. 373. Elsevier, Amsterdam (1980).
17. Y. Cherrah, R. Zini, J. B. Falconnet, M. Desage, J. P. Tillement and J. L. Brazier, *Biomed. Environm. Mass Spectrom.* **14**, 653 (1987).
18. J. B. Falconnet, J. L. Brazier and M. Desage, *J. Label. Compd Radiopharm.* **XXIII**, 267 (1986).
19. M. Desage, J. Soubeyrand, A. Soun and J. L. Brazier, *J. Chromatogr.* **336**, 285 (1984).
20. Y. Benchekroun, B. Ribon, M. Desage and J. L. Brazier, *J. Chromatogr. Chrombio.* **420**, 287 (1987).
21. R. Zini, P. D'Athis, J. Barre and J. P. Tillement, *Biochem. Pharmac.* **28**, 2661 (1979).