Study of Deuterium Isotope Effects on Protein Binding by Gas Chromatography/Mass Spectrometry. Caffeine and Deuterated Isotopomers

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A study of the binding to human serum albumin (HSA) of caffeine and its deuterated isotopomers, 1-C^2H_3-, 3-C^2H_3-, 1,7-$(C^2H_3)_2$-, 3,7-$(C^2H_3)_2$- and 1,3,7-$(C^2H_3)_3$-caffeine, was performed by equilibrium dialysis. Free and bound fractions were measured by gas chromatography/mass spectrometry. Important and significant (Fischer and Student tests) isotope effects were observed on binding parameters: sites total concentration ($N = 1732$ μM for 1,3,7-$(C^2H_3)_3$-caffeine versus 822 μM for caffeine; number of sites ($n = 3$ for 1,3,7-$(C^2H_3)_3$-caffeine v. 1 for caffeine); and extent of binding (46% for 1,3,7-$(C^2H_3)_3$-caffeine v. 27% for caffeine).

A study of competition for HSA binding between caffeine and its 1,3,7-$(C^2H_3)_3$- and 3,7-$(C^2H_3)_2$-isotopomers confirmed the results obtained in direct binding studies. These isotope effects are discussed in terms of (a) tools for molecular pharmacology, (b) precautions to be taken when such labelled drugs are used in clinical pharmacology.

INTRODUCTION

Generally, binding of a given drug to circulating proteins (albumin, alpha$_1$-glycoprotein) modulates the concentration of the free drug (reversible interaction), its diffusion to tissues and consequently its pharmacological activity.[1]

Binding of the drug may determine transient plasmatic retention, selectivity of distribution to certain tissues, or simple transport according to the strength of the underlying forces which depend on the physicochemical properties of the drug (e.g. dissociation constant, cationic or anionic nature at the pH of interest, lipophilicity).

The pharmacological implications of these different incidences of binding are understandably quite different from one another. In order to predict them, one needs to know not only the amount of bound drug but also the intensity of the implicated binding forces in plasma.[2]

For about 15 years, growing interest has been devoted to stable isotope techniques using ^2H,^{13}C,^{15}N and ^{18}O for pharmacokinetic and metabolic studies in man. This is accounted for by the constant development and improvements in equipment available for isotopic detection and measurements from biological samples, particularly gas chromatography/mass spectrometry (GC/MS) and mass fragmentometry.[3–5] Extensive use of stable isotopes is made in clinical pharmacokinetics of normal and pathological states by pulse-dose trials[6] as well as in the elucidation of metabolic pathways using ion-cluster techniques.[7,8] Stable isotopes are also used in bioavailability and bioequivalence studies, placental transfer of drugs and numerous other biological investigation methods (e.g. breath tests, measurement of the body's urea pool, studies on proteic and glucidic metabolisms.[11]

This growing use of stable isotopes permits the extension of the field of applications of these tracers to more specific problems, such as isotope effects. These effects comprise alterations in the behaviour of molecules on substitution by stable or radioactive isotopic atoms,[12–14] affecting both physicochemical and biological parameters such as protein transport, tissue diffusion and metabolism.

An example of such isotope effects is the switching of drug metabolic pathways. Substitution of a C^2H_3 group for a CH_3 group, as in 2-C^2H_3 antipyrin and 1-C^2H_3 caffeine, respectively, enhances demethylation at positions 2 and 7, making these reactions the major metabolic pathways for such deuterated drugs.[15] An increase in the hypnotic activity of butethal deuterated at position 3′[16] and decreased side effects for deuterium-labelled methoxyflurane and halotane[17] can be observed.

It thus appears that, although they may lead to false interpretation of results as far as methodologies requiring pure tracers are concerned, isotope effects may also be exploited with benefit. Considering how large the incidence of isotopic substitution on kinetic or metabolic processes may be, labelling can also be used for explicative purposes[18,19] and for design of better drugs.[20,21]

In the following study, caffeine and its various deuteromethyl isotopomers provide a powerful tool for the demonstration and comparison of isotope effects on protein transport of drugs. The magnitude of isotope effects depends on the molecular position as well as on the nature of the label,[22] which prompted us to study various labelling sites.

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MATERIALS AND METHODS

Structure of caffeine isotopomers

In order to study deuterium isotope effects on the binding of caffeine to human serum albumin (HSA), the following isotopomers were used: unlabelled, 1-C^2H_3, 3-C^2H_3, 1,7-$(C^2H_3)_2$, 3,7-$(C^2H_3)_2$ and 1,3,7-$(C^2H_3)_3$ caffeines (Fig. 1).

Caffeine isotopomers: synthesis and purification

The method used for synthesis of the various isotopomers was that formerly decribed by Falconnet[23] with column chromatographic purification.

Mono-trideuteromethyl analogues (1-C^2H_3 and 3-C^2H_3-caffeine) were obtained from theobromine (3,7-dimethylxanthine, Sigma Chemical Co.) and paraxanthine (1,7-dimethylxanthine, Fluka) respectively.

Di-trideuteromethyl analogues (1,7- and 3,7-$(C^2H_3)_2$-caffeine) were prepared from 3- and 1-methylxanthine (Fluka).

Tri-trideuteromethylcaffeine (1,3,7-$(C^2H_3)_3$-caffeine) was obtained from xanthine (Sigma Chemical Co.).

In all five cases, the procedure adopted was the following: to a stirred solution of 500 mg: of xanthine, mono- or dimethylxanthine in 25 ml acetone: water (1:1) were added successively 25 ml of 0.5 N sodium hydroxide solution and 750 µl (or 500 µl or 250 µl for synthesis of di- and mono-trideuteromethylcaffeine molecules, respectively) C^2H_3I (CEA, Saclay, France); isotopic enrichment: 99.25%.

Additional aliquots of deuteromethyl iodide (750, 500 or 250 µl) were added after 2 h. After a two-day standing at room temperature, acetone was removed under a stream of nitrogen and 50 ml of water added. The rough product was extracted from the aqueous solution using chloroform (3 × 50 ml). The organic solvent was removed in a rotary evaporator (55 °C). Each drug residue obtained was dissolved in methanol and eluted on a silica column (Kieselgel 60, 230–240 mesh) using chloroform at a flow rate of 5 ml min^{-1}. Eluted fractions were collected from 5 to 65 min. Starting and intermediate reaction products are thus retained on top of the chromatographic column. Such a procedure is less time-consuming than thin-layer chromatographic purification, although it provides caffeine analogues of identical chemical and isotopic purities as the former method. After terminal solvent evaporation, labelled caffeine yields ranged from 10 to 60%, with values decreasing from tri- to di- and mono-trideuteromethyl isotopomers. Chemical and isotopic purities were assayed by means of mass spectrometry, proton, deuterium and ^{13}C nuclear magnetic resonance (NMR) and high-performance liquid chromatography (acetic acid: acetonitrile: 2-isopropanol: water, (1:2:3:94).

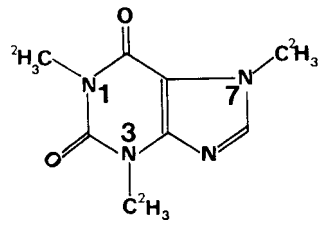

Figure 1. Structure of 1,3,7-$(C^2H_3)_3$-caffeine.

Protein binding studies

Equilibrium dialysis. Caffeine binding was studied by equilibrium dialysis using a Dianorm® apparatus with 20 cells (0.2 × 0.2 ml) under constant stirring (20 rpm). All the experiments were carried out at 37 °C and pH = 7.4 (Sorensen buffer: KH_2PO_4 M/15, $Na_2HPO_4 \cdot 12H_2O$, M/15; constant ionic strength, $\mu = 0.266$ M). In each cell, the two compartments are separated by a semipermeable cellulose membrane (Visking, diameter 15–20 Å) which retains compounds with molecular weights over 12 000. In initial experiments, no significant binding of the drug through the membrane and on cell walls was observed. All subsequent experiments were performed for 3 h. Increasing concentrations of caffeine and deuterocaffeines (50–15 000 µM) were dialysed against a fixed protein concentration (HSA = 600 µM). Each deuteromethylcaffeine dialysis trial was performed along with unlabelled caffeine dialysis. At the end of the dialysis process, drug concentration in each compartment was measured by GC/MS.

Competition studies: protein binding of caffeine in the presence of deuterated isotopomers. A possible inhibition of caffeine binding to HSA was investigated using 3,7-$(C^2H_3)_3$-caffeine (final concentration 3000 µM) or 1,3,7-$(C^2H_3)_3$-caffeine (final concentration 2000 µM). Here too, caffeine binding was studied in the concentration range 50–15 000 µM (phosphate buffer pH = 7.4, 37°C, 3 h).

Caffeine and deuteromethylcaffeine assay

Following equilibrium dialysis, free and bound caffeine and deuteromethylcaffeine concentrations were determined in duplicate by means of GC/MS according to a method derived from theophylline assay.[24]

50-µl samples recovered from each cell are added with 50 µl internal standard solution and extracted with 1.5 ml of chloroform: isopropanol mixture (95:5 v/v) in 200 µl acetate buffer (pH = 5.2). Internal standard is either $^{15}N_{1-3}$, $^{13}C_2$ caffeine (mol. wt = 197) for assays of unlabelled caffeine (mol. wt = 194), di-trideuteromethyl (mol. wt = 200), and tri-trideuteromethyl caffeines (mol. wt = 203); or natural caffeine for mono-trideuteromethylcaffeines (mol. wt = 197) assays. In practice, 50 µl of solutions containing 200 mg l^{-1} or 2 g l^{-1} of internal standard are used, depending on the range of caffeine concentrations to be measured.

After extraction, each dry residue is redissolved in 100 µl of a toluene: ethyl acetate (5:2 v/v) mixture and 1 µl of the subsequent solution injected into the chromatograph: HP 5790, OV-1701 capillary column, splitless mode; injection port temperature 230 °C, single column temperature ramp 130–235 °C, 15 °C min^{-1}.

An HP 5970A quadrupolar mass selective detector permits quantification of selected ions, namely molecular ions: $m/z = 194$ (caffeine), $m/z = 197$ (mono-trideuteromethylcaffeine), $m/z = 200$ (di-trideuteromethylcaffeine) and $m/z = 203$ (tri-trideuteromethylcaffeine).

Calculation of HSA binding parameters

Following equilibrium dialysis, free (F) and bound (B) fractions are measured and B is plotted vs. F according to equation:

$$B = NK_aF/(1+K_aF) = nPK_aF/(1+K_aF)$$

where P is the total protein concentration.

In cases where protein binding is a saturable process, the association constant (K_a), as well as total sites concentration (N) and number of sites (n), are readily calculated from this curve. In our study, HSA binding parameters were obtained from the Scatchard plot:

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

using a Tektronix 4051 computer. As concerns data obtained in the presence of binding inhibitors, they were processed in two successive steps by drawing B vs. F and Scatchard plot, first for caffeine alone and secondly for caffeine in the presence of each inhibitor.

Statistical analysis of data

Student's t-test was employed for comparison of HSA binding parameters of natural caffeine and deuterated analogues. For determining caffeine HSA binding model and competition studies, Fisher's test was used.

RESULTS

Protein binding of unlabelled caffeine

A preliminary binding experiment using human serum adjusted to 70 g l^{-1} proteins, 40 g l^{-1} of which were HSA, revealed the presence of endogenous caffeine inhibitors on HSA binding sites. At 50 μM concentration, caffeine binding to whole serum was 19.8%, as opposed to 27.1% for HSA alone.

Binding of caffeine to HSA appears to follow a saturable process (Fig. 2). The B fraction decreases from 27% to 4.1% as drug concentrations are increased from 50 to 15 000 μM. This binding displays a small affinity

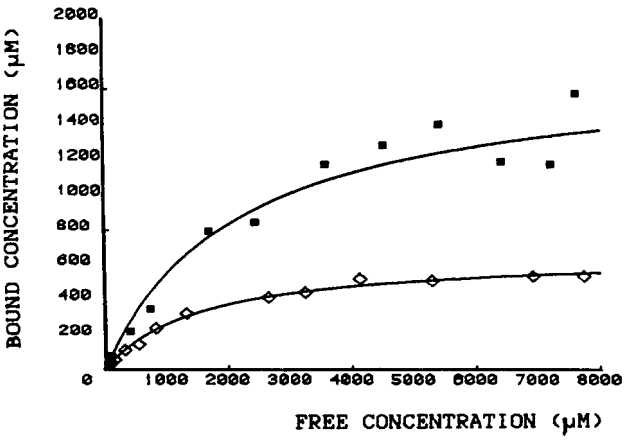

Figure 2. B vs. F plot for unlabelled caffeine (◇) and its 1,3,7-$(C^2H_3)_3$ isotopomer (■).

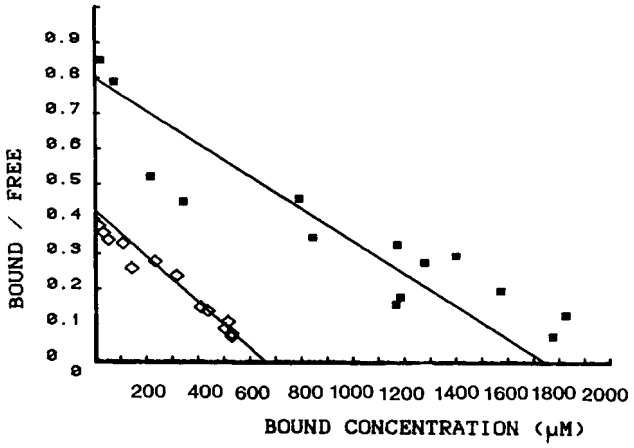

Figure 3. Scatchard plot: B/F vs. B for unlabelled caffeine (◇) and its 1,3,7-$(C^2H_3)_3$ isotopomer (■).

constant ($K_a = 486\ M^{-1}$) and a single class of binding sites ($n = 1$) (Fig. 3).

Protein binding of deuterated caffeines

The parameters N, K_a and percentage binding are given in Table 1. As for natural caffeine, HSA binding of all five deuteromethyl analogues is a saturable process (Fig. 2) with a single class of binding sites (Fig. 3). However, the percentage binding of 1,3,7-$(C^2H_3)_3$-, 3,7-$(C^2H_3)_2$- and 3-C^2H_3-caffeine is greater than that for natural caffeine. Moreover, the total site concentration (N) of all five deuterated analogues exceeds that of the unlabelled product (ranging from 1732 μM for the tri-trideuteromethyl analogue to 822 μM for natural caffeine). It can be observed also that the number of binding sites increases with deuteration, from one site for caffeine to three sites for tri-trideuteromethylcaffeine.

Lastly, the association constant (K_a) decreases in di-trideuteromethyl analogues, whereas mono- and tri-trideuteromethyl analogues show no significant K_a alteration when compared to natural caffeine.

Competition studies

The percentage of caffeine binding in the presence of 3,7-$(C^2H_3)_2$ and 1,3,7-$(C^2H_3)_3$-caffeines is given in Table 2. It appears that competition significantly reduces HSA

Table 1. Binding parameters of caffeine and its isotopomers

Isotopomers	N Mean	N S.D.	n	K_a Mean	K_a S.D.	%
Caffeine	822	80	1.00	486	35	27.00
Caffeine 1CD3	1110	76	2.00	405	74	27.50
Caffeine 3CD3	1209	70	2.00	362	55	33.10
Caffeine 1-7(CD3)2	1159	50	2.00	284	25	27.00
Caffeine 3–7(CD3)2	1494	78	2.50	267	44	42.00
Caffeine 1-3-7(CD3)3	1732	176	3.00	460	17	46.00

N = Total concentration of binding sites (μM).
n = Number of binding sites.
K_a = Affinity constant (M^{-1}).
% = Percentage binding for a 50 μM concentration.

Table 2. Binding parameters of caffeine and in the presence of two isotopomers

Isotopomers	N Mean	N S.D.	n	K_a Mean	K_a S.D.	%
Caffeine	822	80	1.00	486	35	27.00
Caf./caf 3-7(CD3)2	444	31	0.74	268	40	12.50
Caf./caf 1-3-7(CD3)3	535	69	0.89	341	109	13.30

N = Total concentration of binding sites (μM). n = Number of binding sites. K_a = Affinity constant (M^{-1}). % = Percentage binding for a 50-μM concentration.

caffeine binding. Competition also decreases caffeine total site concentration (Figs 4 and 5).

DISCUSSION

Differences in physicochemical properties such as pK_a and lipophilicity between labelled and unlabelled molecules are responsible for biological isotope effects[14] which may affect pharmacokinetic, pharmacodynamic and toxic steps. Variations in lipophilicity lead to altered chromatographic behaviour of deuterated isotopomers as compared to their natural counterpart.[26]

A comparable effect can be observed in Fig. 6, where 1,3,7-$(C^2H_3)_3$ caffeine is retained to a lesser degree on the chromatographic column (ion $m/z = 203$) than natural caffeine (ion $m/z = 194$) and $^{15}N_{1-3}$,$^{13}C_2$-caffeine (ion $m/z = 197$).

Since binding to HSA mainly depends upon the physicochemical properties of molecules (hydrogen and hydrophobic bonds), it is not surprising to observe HSA binding alterations through deuterium substitution, the magnitude of which is conditioned both by the number of deuterium-substituted sites in the molecule and the degree of implication of these sites in the binding process: a major isotope effect occurs for deuterium substitution at the site that binds HSA.

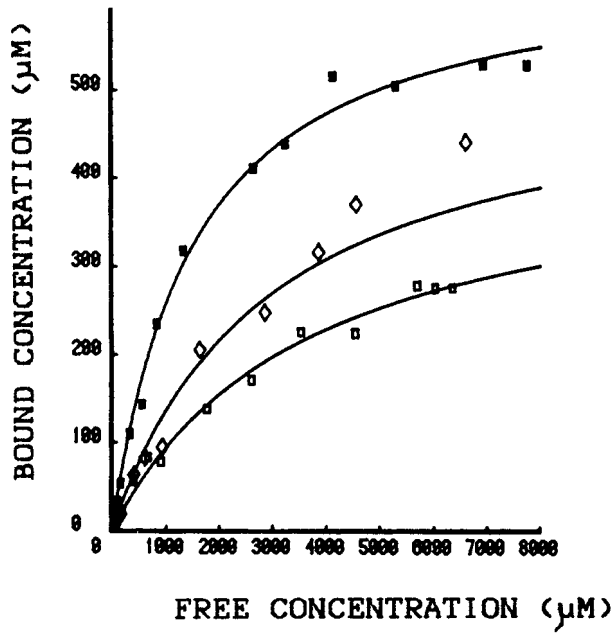

Figure 4. Competition study: B. vs. F plot for: (■) unlabelled caffeine, (◇) caffeine in the presence of its 1,3,7-$(C^2H_3)_3$ isotopomer, (□) caffeine in the presence of its 3,7-$(C^2H_3)_2$ isotopomer.

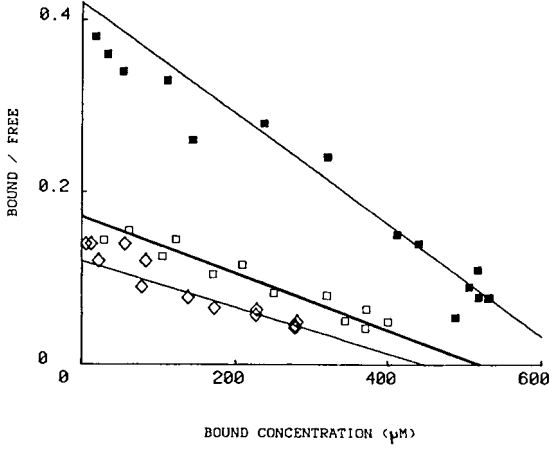

Figure 5. Competition study: Scatchard plot, B/F vs. B for: (■) unlabelled caffeine, (◇) caffeine in the presence of its 1,3,7-$(C^2H_3)_3$ isotopomer, (□) caffeine in the presence of its 3,7-$(C^2H_3)_2$ isotopomer.

The caffeine isotopomer model demonstrates that differences in HSA binding due to isotope effects may be as large as 100% (46% for tri-trideuteromethyl-caffeine v. 27% for natural caffeine), a result confirmed by competition studies using two isotopomers for caffeine displacement from its HSA binding sites.

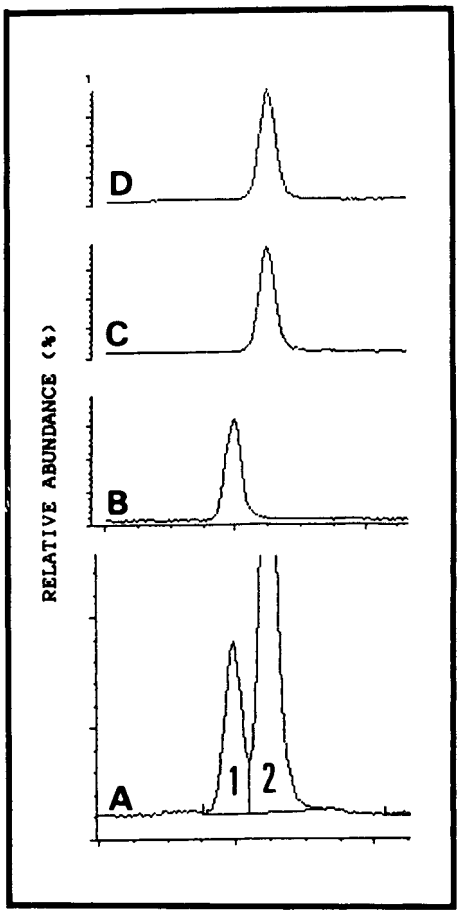

Figure 6. Chromatographic isotopic effect: chromatogram of a mixture of unlabelled caffeine (I), $^{15}N_{1-3}$,$^{13}C_2$-caffeine (II) and 1,3,7-$(C^2H_3)_3$-caffeine (III). For conditions see text. A, total ion current: 1 = (III) − RT = 10.19 min; 2 = (I + II) − RT = 10.24 min; B, trace for molecular ion m/z = 203 from (III); C, trace for molecular ion m/z = 197 from (II); D, trace for molecular ion m/z = 194 from (I).

Such variations in drug binding may have considerable incidence *in vivo*, since drug-free fraction varies in inverse proportion to the fraction bound and the sole free fraction is considered pharmacologically active. Reduction in effectiveness or increased toxicity may thus ensue from deuterium labelling, especially in the case of drugs with low therapeutic index.

At last, one must stress that, although significant effects are associated with deuterium substitution as in the case of caffeine, isotope effects may theoretically be even greater through molecular substitution by heavier isotopes such as tritium.[14]

CONCLUSION

Deuterium isotopic labelling induces isotope effects on HSA binding of caffeine, the magnitude of which is conditioned by the number as well as the nature of labelled sites. Such local perturbation studies provide valuable information concerning the position of the binding site in the ligand molecule (which dynamic NMR studies may alternatively help precise). However, deuterium substitution may modify the drug-free (active) fraction through alterations in the binding percentage and sites concentration.

Adequate choice of labelling atom and site therefore appears necessary to avoid isotope effects in the course of studies requiring true tracers.

On the other hand, labelling of various molecular sites may be used intentionally, isotopic substitution being here a means of producing local successive molecular perturbations that may help uncover the intimate mechanisms underlying biological processes.

In this way, isotope effects may provide very useful tools for molecular biology in the coming years.

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