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INTERACTIONS WITH THE PROTEIN BINDING OF 7-HYDROXY-METHOTREXATE IN HUMAN SERUM *IN VITRO*

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Abstract—7-Hydroxy-methotrexate (7-OH-MTX), the major extracellular methotrexate (MTX) metabolite, is 90–95% bound in human serum, with albumin (HSA) as the major binding protein. Reports of an interaction with concomitantly administered non-steroidal antiinflammatory drugs (NSAIDs) during MTX therapy led us to investigate whether these compounds could reduce the binding of 7-OH-MTX *in vitro*. Equilibrium dialysis experiments demonstrated that naproxen and indomethacin concentration dependently reduced the binding of 1 μM 7-OH-MTX. After ingestion of 1000 mg naproxen, per cent unbound 7-OH-MTX in sera from volunteers increased 2–3-fold *in vitro*, positively correlated to naproxen concentrations ($P < 0.00015$). In addition, etacrynic acid, bilirubin, sulphamethizole and acetylsalicylic acid displaced 7-OH-MTX from its binding protein(s) in a competitive manner. The data suggest that 7-OH-MTX interacts with several exogenous and endogenous substances associated with HSA in human serum. Displacement of 7-OH-MTX from HSA may contribute to the interaction between NSAIDs and MTX.

7-Hydroxy-methotrexate (7-OH-MTX),† the major extracellular metabolite of the antifolate agent methotrexate (MTX), is measured in relatively high concentrations in the blood following high-dose MTX therapy [1–3]. 7-OH-MTX appears to have a lower volume of distribution than the parent compound [3], and has been found to be 90–95% bound in human serum at therapeutic concentrations [4, 5], with serum albumin (HSA) as the primary binding molecule [5, 6]. Compared to MTX, which is 40–50% bound mainly to HSA in serum [7–9], this would make 7-OH-MTX a more likely candidate for quantitatively important interactions to occur on the level of protein binding.

Coadministration of non-steroidal antiinflammatory drugs (NSAIDs) and MTX has previously led to toxic and sometimes fatal manifestations in patients [10–13]. Although the interaction between NSAIDs and MTX has been proposed to be caused by the inhibitory effect of NSAIDs on renal prostaglandin synthesis [11, 12], the mechanism has remained a matter of speculation. 7-OH-MTX is cytotoxic [14, 15], and has been proposed as a mediator of renal toxicity following high-dose MTX [16, 17]. Since both 7-OH-MTX and NSAIDs are highly bound to HSA in serum, and displacement of 7-OH-MTX by the latter from binding sites would increase the concentration of 7-OH-MTX eligible for renal filtration, this could contribute to renal damage. To elucidate whether binding of 7-OH-MTX in serum was affected by the presence of NSAIDs and other drugs which bind to HSA, equilibrium dialysis experiments with 7-OH-MTX, using serum spiked with different concentrations of 10 drugs, or sera from volunteers after administration of naproxen, were undertaken. Besides two compounds of the NSAID class, some commonly used drugs which are highly protein bound, bilirubin, leucovorin, and the non-protein bound agent allopurinol, were tested.

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† Abbreviations: 7-OH-MTX, 7-hydroxy-methotrexate; MTX, methotrexate; HSA, human serum albumin; NSAIDs, non-steroidal antiinflammatory drugs.

MATERIALS AND METHODS

Chemicals. 7-OH-MTX was obtained by preparative high pressure liquid chromatography (HPLC) of urine from a patient given intravenous high-dose MTX therapy. Chromatography was performed essentially according to a method previously described [18], but with fraction sampling, and further purification by HPLC using a mobile phase of distilled water, pH 6.2, and subsequent washout of retained substance with methanol and water (50:50, v/v). After freeze-drying and resuspension, HPLC showed a single peak, identical with 7-OH-MTX obtained from Dr W. E. Evans, St. Jude Children's Research Hospital, Memphis, TN, U.S.A.

Bilirubin was from Sigma Chemical Co. (St Louis, MO). Drugs were obtained from the following sources: Indomethacin, Merck & Co. Inc. (Rahway, NJ); allopurinol, Sigma Chemical Co. (St Louis, MO); digitoxin, NAF-Laboratoriene A/S (Oslo, Norway); and leucovorin (calcium folinate), A/S Nycomed (Oslo, Norway). Naproxen (A/S Nycomed, Oslo, Norway), phenylbutazone (Løvens kemiske Fabrik, Copenhagen, Denmark), etacrynic acid (Merck & Co. Inc., Rahway, NJ), and acetylsalicylic acid and sulphamethizole (NAF-Laboratoriene A/S, Oslo, Norway) were kindly

donated by the suppliers. All other reagents were of analytical grade.

Serum. Serum was obtained from venipuncture of a 31-year-old, healthy male, who took no drugs. Six healthy, drug-free volunteers, 3 females and 3 males (mean age 34 years, range 25–43 years) were given an oral dose of 4 × 250 mg naproxen tablets (Naprosyn®, Astra-Syntex Scandinavia AB, Södertalje, Sweden) after an overnight fast. Blood samples were obtained immediately prior to drug ingestion and 2 and 6 hr afterwards.

Serum was prepared by leaving venous samples at room temperature for 1 hr prior to centrifugation at 2000 *g* for 10 min. Prior to experiments, serum was stored at −20° for a maximum of 2 weeks.

Protein binding. Binding of 7-OH-MTX in serum was determined by equilibrium dialysis, using a dialysis membrane 32/32 (Medicell International Ltd., London, U.K.), clamped between two Perspex® cells. 7-OH-MTX (50 μl in 0.9% saline) was added to 450 μl serum, in absence or presence of displacer, to achieve a final concentration of 1 μM. The displacing compounds were dissolved in 0.9% saline or in 0.1 M Na_2CO_3 before the sera were spiked. Equal amounts of 0.1 M Na_2CO_3 did not affect the binding of 7-OH-MTX in serum in separate experiments (data not shown). The samples were dialyzed against 500 μl of Krebs Ringer bicarbonate buffer [19] at 37°, in an atmosphere of air with 10% CO_2, with gentle shaking, and protected

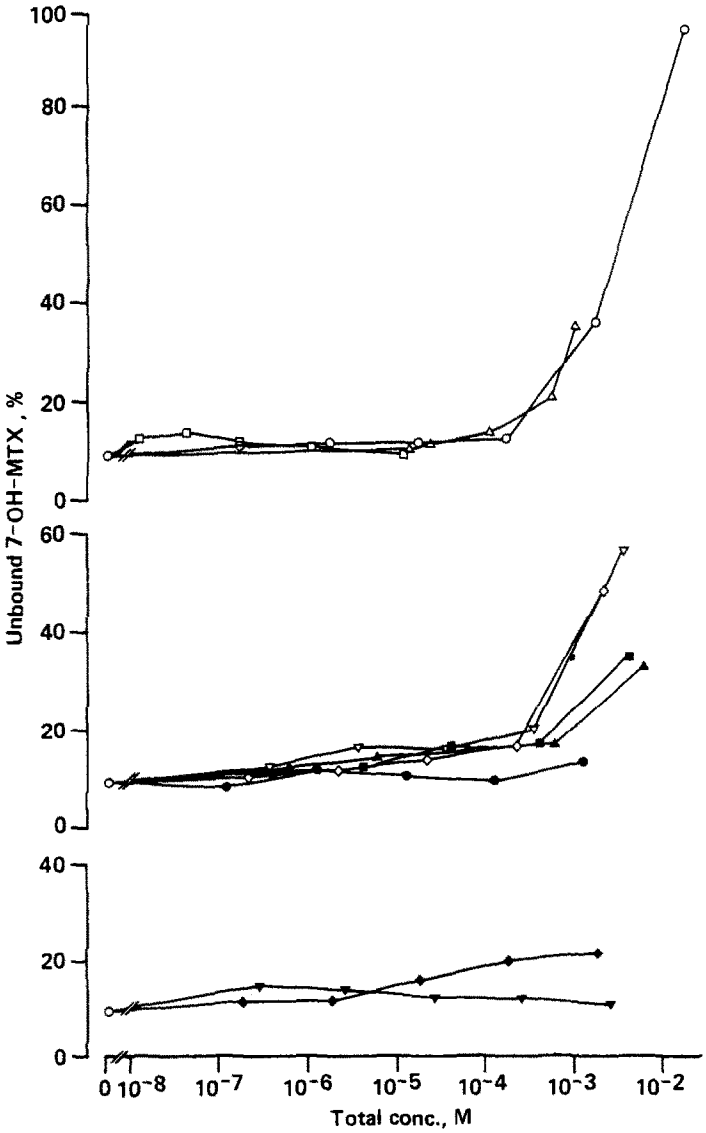

Fig. 1. Percent unbound 7-hydroxy-methotrexate (7-OH-MTX) as a function of increasing the total added concentration of various compounds in equilibrium dialysis experiments with 10^{-6} M 7-OH-MTX. (a) Upper part: indomethacin (○), bilirubin (△), digitoxin (□). (b) Middle part: naproxen (◇), ethacrynic acid (▽), phenylbutazone (●), sulphamethizole (■), acetylsalicylic acid (▲). (c) Lower part: leucovorin (◆), allopurinol (▼). Each point on the curve represent the mean of 3–4 experiments, with SD < 5%.

Table 1. Interaction with serum protein binding of 10^{-6} M 7-OH-MTX by various substances

Compound	No. of experiments	Conc. range (μM)	pH	Conc. of compound (μM) to increase %F of 7-OH-MTX 100%	200%	300%	400%
Indomethacin	23	0.14–14000	7.35 ± 0.10	240	580	1400	2000
Bilirubin	23	11–852	7.53 ± 0.10	240	600	—	—
Digitoxin	19	0.01–9.36	7.47 ± 0.11	—	—	—	—
Naproxen	18	0.2–2000	7.58 ± 0.16	220	420	840	1600
Ethacrynic acid	19	0.33–3300	7.43 ± 0.09	96	520	930	1600
Phenylbutazone	20	0.11–1100	7.39 ± 0.09	—	—	—	—
Sulphamethizole	18	0.37–3700	7.43 ± 0.10	420	1400	—	—
Acetylsalic acid	18	0.55–5500	7.39 ± 0.08	660	2500	—	—
Leucovorin	18	0.17–1700	7.52 ± 0.15	94	—	—	—
Allopurinol	20	0.24–2400	7.59 ± 0.24	—	—	—	—

Each compound was added to serum in 5 or 6 different concentrations, and the number of experiments and the total concentration range investigated for each substance is given. pH is given as mean ± SD values of measurements in both serum and buffer from all experiments. In non-supplemented serum, percent unbound (%F) 7-OH-MTX was 9.2 ± 3.4% at pH 7.46 ± 0.08 (N = 10). To the right, the concentrations of added compound which increased %F by 100% increments, as read from the curves (Fig. 1), are given.

from light. Following removal from the dialysis cells, pH in both serum and buffer was immediately measured (Radiometer, Copenhagen, Denmark), and the samples were analyzed on the same day. Equilibrium was reached within 16 hr, was unaltered for the following 8 hr, and was independent of whether 7-OH-MTX was added to the serum or the buffer side of the dialysis membrane (data not shown). Recovery of 7-OH-MTX from the dialysis cells was complete (data not shown).

Determination of 7-OH-MTX, naproxen, and serum albumin. Serum and buffer concentrations of 7-OH-MTX were measured by HPLC [18]. Naproxen concentrations in serum were measured by HPLC, using a method described in detail elsewhere [20]. Serum albumin (HSA) concentrations were determined by the bromcresol-green method according to Boehringer Mannheim automated analysis for BM/Hitachi system 737 (Dec. 1984 edition).

Calculations. Statistical computations were performed using Microstat®, a microcomputer program from Ecosoft Inc. (Indianapolis, IN).

RESULTS

The results of the equilibrium dialysis experiments using spiked serum samples from a single subject, with a HSA concentration of 48.0 g/l, are presented in Fig. 1 and in Table 1. Of the substances tested, indomethacin, bilirubin, naproxen and ethacrynic acid were the most potent in terms of reducing binding of 10^{-6} M 7-OH-MTX. Leucovorin, sulphamethizole and acetylsalicylic acid reduced the protein binding to a lesser degree, while digitoxin, phenylbutazone and allopurinol did not alter the binding properties of 7-OH-MTX.

After administration of 1000 mg naproxen, sera from the 6 volunteers, where albumin concentrations were normal (46.7 ± 2.4 g/l, mean ± SD), revealed a decrease in binding of 10^{-6} M 7-OH-MTX. In serum samples obtained before ingestion of the drug, percent unbound drug (%F) was 9.5 ± 2.7%. After 2 hr the %F increased in all subjects to 22.9 ± 4.8% with naproxen concentrations of 373 ± 72 μM. At 6 hr the %F was 18.5 ± 7.6% and the naproxen concentrations in the samples were 230 ± 31 μM (mean ± SD). Compared to zero values, the 2 and 6 hr increases in %F 7-OH-MTX were both significant (Wilcoxon signed rank test, $P < 0.015$), while there was no significant difference between the 2 and 6 hr sera in terms of 7-OH-MTX binding ($P > 0.05$). %F of 7-OH-MTX was closely correlated with naproxen concentrations ($y = 0.037x + 9.53$, $r = 0.78$, $P < 0.00015$) (Fig. 2).

DISCUSSION

HSA possesses several qualitatively different binding domains for drugs and endogenous compounds [21–23]. Indomethacin, naproxen, phenylbutazone, ethacrynic acid and probably sulphamethizole appear to bind to the indole site, or to subgroups of this site, on HSA [21, 22]. A digitoxin binding site has been identified [22], but the relationship of this site to the site for indomethacin is uncertain [23]. Bilirubin binds to the bilirubin site on HSA [22]. Specific HSA binding sites for leucovorin, and acetylsalicylic acid, has to our knowledge not been localized. Allopurinol does not bind to proteins in serum [24].

Naproxen reduced the binding of 7-OH-MTX in the same manner as indomethacin, and so did the diuretic agent ethacrynic acid. Equimolar addition of both indomethacin and bilirubin to serum gave a reduction in 7-OH-MTX binding, thus demonstrating that 7-OH-MTX seem to bind to both indole and bilirubin sites. Digitoxin concentrations up to 9.36 μM did not affect the binding of 7-OH-MTX in serum. Sulphamethizole, representing the sulphonamide group of drugs, is highly bound to HSA [24]. The lesser reduction of the binding of 7-OH-MTX in serum by sulphamethizole as compared to indomethacin or naproxen can be caused by the comparably lower degree of binding of this drug *per se*. The smaller reduction in bound 7-OH-MTX in the presence of acetylsalicylic acid may likewise reflect that this drug is less protein bound, especially at high

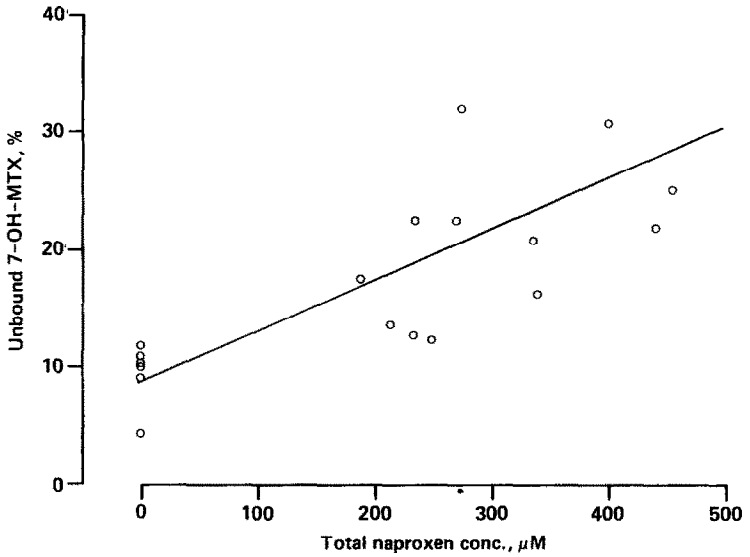

Fig. 2. Correlation between percent unbound 7-hydroxy-methotrexate (7-OH-MTX) and total naproxen concentration in serum samples from 6 healthy volunteers. Each volunteer ingested 1000 mg naproxen, and samples were obtained before and 2 and 6 hr after administration of the drug. pH in serum and buffer samples was 7.31 ± 0.09, and each point represent the mean of two experiments. See text for details.

concentrations [25]. Surprisingly, phenylbutazone did not reduce the binding of 7-OH-MTX to any great extent. The observation that leucovorin increased the free fraction of 7-OH-MTX 2-fold at a concentration of approximately 100 μM, suggests an interaction with 7-OH-MTX binding sites on HSA. Leucoverin did, however, not further displace the binding of 7-OH-MTX at higher concentrations.

Experiments with sera from volunteers were undertaken to elucidate whether binding of 7-OH-MTX could be affected by therapeutic concentrations of a NSAID, and naproxen was chosen because of its properties as a potent displacer after *in vitro* spiking of samples. Unbound 7-OH-MTX in sera before ingestion of the drug, 9.5 ± 2.7%, was of the same magnitude as reported earlier [4, 5], but after administration of naproxen, per cent unbound 7-OH-MTX increased 2–3-fold, positively correlated with serum naproxen concentrations (Fig. 2), which were of the same magnitude as encountered clinically [26].

7-OH-MTX is highly bound to HSA in the blood [4–6]. The present study demonstrates that exogenous and endogenous substances with affinity for different binding sites or domains on HSA, such as indomethacin and bilirubin, or compounds with affinity for different subdomains on the same binding site, such as indomethacin and ethacrynic acid, reduce the serum binding of 7-OH-MTX in serum in the same manner. Whether this reflects affinity for 7-OH-MTX at multiple HSA sites, or is a consequence of a conformational change in the HSA molecule, is unknown.

Acidic drugs with low volumes of distribution which are also extensively bound to HSA, such as indomethacin and naproxen, may increase unbound 7-OH-MTX in serum severalfold. Whether the increase in free 7-OH-MTX enhances toxicity, either by precipitation in renal tissue, or by other mechanisms of action, remains obscure. In general, two categories of patients are prone to develop renal damage with NSAID therapy alone; those with decreased effective circulatory volume and those with compromised renal function [27]. MTX is nephrotoxic in its own right [28]. A substantial increase in renal filtration of the active and, by comparison, relatively insoluble metabolite would greatly increase the concentration of 7-OH-MTX in renal tissue, consistent with the theory that tissue damage is concentration-dependent and likely to occur in areas with the greatest drug concentration. Our results suggest that the pronounced reduction in bound 7-OH-MTX, caused by drugs like NSAIDs, may be of importance for the observed interaction between MTX and these drugs in the clinic.

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REFERENCES

1. H. Breithaupt and E. Kuenzlen, *Cancer Treat. Rep.* **66**, 1733 (1982).
2. B. Winograd, R. J. J. Lippens, M. J. M. Oosterbaan, M. J. M. Dirks, T. B. Vree and E. van der Kleijn, *Eur. J. clin. Pharmac.* **30**, 231 (1986).
3. L. Slørdal, S. Kolmannskog, P. S. Prytz, P. J. Moe and J. Aarbakke, *Pediatr. Hematol. Oncol.* **3**, 127 (1986).
4. H. Breithaupt, E. Kuenzlen and G. Goebel, *Anal. Biochem.* **121**, 103 (1982).
5. L. Slørdal, G. Sager, N.-E. Huseby and J. Aarbakke, *J. Pharm. Pharmac.* in press.
6. C. Lopez, M. N. Bordeaux, M. Chauvet, R. Gilli and C. Briand, *Biochem. Pharmac.* **35**, 2834 (1986).
7. W. H. Steele, J. R. Lawrence, J. F. B. Stuart and C. A. McNeill, *Eur. J. clin. Pharmac.* **15**, 363 (1979).

8. J. W. Paxton, *J. Pharmac. Meth.* **5**, 203 (1980).
9. J. W. Paxton, *Clin. exp. Pharmac. Physiol.* **9**, 225 (1982).
10. N. M. Ellison and R. J. Servi, *Cancer Treat. Rep.* **69**, 342 (1985).
11. A. Thyss, G. Milano, J. Kubar, M. Namer and M. Schneider, *Lancet* **i**, 256 (1986).
12. A. G. Maiche, *Lancet* **i**, 1390 (1986).
13. R. R. Singh, A. N. Malaviya, J. N. Pandey and J. S. Guleria, *Lancet* **i**, 1390 (1986).
14. G. Fabre and I. D. Goldman, *Cancer Res.* **45**, 80 (1985).
15. I. Fabre, G. Fabre and J. P. Cano, *Eur. J. Cancer clin. Oncol.* **22**, 1247 (1986).
16. S. A. Jacobs, R. G. Stoller, B. A. Chabner and D. G. Johns, *J. clin. Invest.* **57**, 534 (1976).
17. S. A. Jacobs, R. G. Stoller, B. A. Chabner and D. G. Johns, *Cancer Treat. Rep.* **61**, 651 (1977).
18. L. Slørdal, P. S. Prytz, I. Pettersen and J. Aarbakke, *Ther. Drug Monit.* **8**, 368 (1986).
19. P. P. Cohen, in *Manometric Techniques and Tissue Metabolism* (Eds. W. W. Umbreit, R. H. Burris and J. F. Stauffer), p. 118. Burgess, Minneapolis (1957).
20. A. Høylandskjær and J. Aarbakke, *Acta Pharmac. Toxic.* **52**, 78 (1983).
21. G. Sudlow, D. J. Birkett and D. N. Wade, *Molec. Pharmac.* **11**, 824 (1975).
22. K. J. Fehske, W. E. Muller and U. Wollert, *Biochem. Pharmac.* **30**, 687 (1981).
23. D. J. Birkett and S. Wanwimolruk, in *Protein Binding and Drug Transport* (Eds. J.-P. Tillement and E. Lindenlaub), pp. 11–23. F. K. Schattauer Verlag, New York (1986).
24. A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad (Eds.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. Macmillan, New York (1985).
25. G. S. Avery (Ed.), *Drug Treatment*. Adis Press, New York (1980).
26. R. S. Porter, *Drug Intell. Clin. Pharm.* **18**, 42 (1984).
27. T. I. Poirier, *Drug Intell. Clin. Pharm.* **18**, 27 (1984).
28. J. Jolivet, K. H. Cowan, G. A. Curt, N. J. Clendeninn and B. A. Chabner, *New Engl. J. Med.* **309**, 1094 (1983).