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Distribution of 7-hydroxymethotrexate in human blood

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Abstract—We have examined the in-vitro distribution of 7-hydroxymethotrexate (7-OH-MTX), a cytotoxic metabolite of methotrexate (MTX), in human blood, and its protein binding in serum. The distribution of 7-OH-MTX (10^{-6} M) in fresh samples of whole blood was studied at 37 °C and pH 7·51 ± 0·05 (mean ± s.d.), and its protein binding was assessed by equilibrium dialysis of serum against Krebs Ringer phosphate buffer at 37 °C and pH 7·41 ± 0·07 (mean ± s.d.). 7-OH-MTX had a mean cell/plasma concentration ratio of 0·03 (range 0–0·27, n = 18). It was extensively bound in human serum, with a bound fraction of 90·4 ± 3·3% (mean ± s.d.) in healthy volunteers (n = 11), and significantly lower, 82·3 ± 4·0% (mean ± s.d.), in hypoalbuminaemic surgical patients (n = 7). The binding of 7-OH-MTX was correlated with serum albumin (HSA) concentrations (r = 0·72, $P < 0·0007$, n = 18). Blood distribution data support the contention that 7-OH-MTX has a small volume of distribution, and HSA appears to be mainly responsible for the high degree of its protein binding in serum.

After high-dose intravenous therapy with the antineoplastic agent methotrexate (MTX), high concentrations of the metabolite 7-hydroxymethotrexate (7-OH-MTX) are detected in the blood (Breithaupt & Kuenzlen 1982; Slørdal et al 1986a). The distribution of any drug in the body is a function of lipophilicity and binding (Øie 1986). Unlike MTX, the distribution and protein binding of which have been extensively studied, little information is available on 7-OH-MTX. We have examined 7-OH-MTX distribution in whole blood and its binding in human serum. As important serum binding proteins, such as albumin (HSA) and α_1-acid glycoprotein (AAG) are recognized to fluctuate with certain situations and diseases (Tillement et al 1978; Øie 1986), the study was carried out with samples from participants where substantial variation in these blood constituents were expected.

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 Slørdal et al (1986b), but with fraction sampling, and further purification by HPLC using a mobile phase of distilled water, pH 6·2, and subsequent wash-out of retained substance with methanol and water (50:50, v/v). After freeze-drying and resuspension, the product on HPLC showed a single peak, identical with 7-OH-MTX obtained from Dr W. E. Evans, St Jude Children's Research Hospital, Memphis, TN, USA. All other reagents were of analytical grade.

Whole blood and serum. Blood was obtained by venipuncture of 18 volunteers, 10 females and 8 males, aged 25–83 years, with a mean age of 48 years. Eleven were healthy, and seven had recently undergone major surgery of the hip. For experiments using whole blood, samples were drawn in EDTA tubes (Vacutainer, Grenoble, France). Serum was prepared by leaving venous samples at room temperature (20 °C) for 1 h before centrifugation at 2000*g* for 10 min.

Distribution in whole blood in-vitro. 200 µL of a solution of 7-OH-MTX in 0·9% NaCl (saline) was added to 1·8 mL aliquots of freshly obtained EDTA blood, to a concentration of 10^{-6} M. In open polyethylene tubes, the samples were gently shaken at 37 °C in an atmosphere of air containing 5% (v/v) CO_2. Initial studies had established that the cell/plasma (C/P) distribution ratio of 7-OH-MTX did not vary with time from 20 to 120 min (data not shown). After 30 min incubation, the samples were centrifuged at 1000*g* and 37 °C for 10 min, using a bench type cooling centrifuge (Sigma 2KD, Sigma Laborzentrifugen GmbH, Osterode am Harz, FRG) fitted with a thermistor, heating device and regulator capable of maintaining the temperature inside the centrifuge bowl within ±0·5 °C of preset values. The samples were then transferred to new tubes, and pH was measured (Radiometer, Copenhagen, Denmark). Calculation of the cellular concentrations of 7-OH-MTX was based on the haematocrit (Cellokrit 2, Linson Instrument AB, Stockholm, Sweden) in the samples, the concentrations of 7-OH-MTX in reference samples (1·8 mL of EDTA plasma samples spiked with the same amount of 7-OH-MTX as the whole blood samples) and 7-OH-MTX

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concentrations in plasma samples after centrifugation. Concentrations in blood cells were obtained by the equation

$$C = R/H - P \times (1 - H)/H$$

where R, C and P are the drug concentrations in reference samples, cellular components, and in plasma after centrifugation, respectively, and H the haematocrit of the samples. Each experiment was performed four times.

Protein binding. Binding of 7-OH-MTX in serum was determined by equilibrium dialysis, using a dialysis membrane 32/32 (Medicell International Ltd, London, UK), clamped between two Perspex cells. 50 µL of 7-OH-MTX in saline was added to 450 µL serum, to a concentration of 10^{-6} M. The samples were dialysed against 500 µL of Krebs Ringer bicarbonate buffer (Cohen 1957) at 37 °C, in an atmosphere of air with 10% CO_2, and with gentle shaking. Equilibrium was reached within 16 h, was unaltered for the following 8 h, 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 were 100·4 ± 4·4% (mean ± s.d., n = 8), demonstrating that no spontaneous decomposition or binding of 7-OH-MTX to the dialysis membrane occurred. Protein binding was expressed as per cent bound (%B), per cent unbound (%F), or as the bound/unbound (B/F) concentration ratio of 7-OH-MTX. Each experiment was performed four times.

Determination of 7-OH-MTX concentrations. Plasma, serum, and buffer concentrations of 7-OH-MTX were measured by HPLC (Slørdal et al 1986b).

Determination of serum proteins, lipids and folate. Serum albumin (HSA) concentrations were determined by the bromcresol green method, and the concentrations of total proteins (TP) were determined by the Biuret reaction, both according to Boehringer Mannheim automated analysis for BM/Hitachi system 737 (Dec 1984 edition). Concentrations of AAG were determined in diluted plasma (9%, v/v) by spectrophotometry (340 nm) after precipitation with anti-AAG (Orion Diagnostica, Espoo, Finland) in 5% (v/v) polyethylene glycol 6000 (E. Merck, Darmstadt, FRG). Triglyceride (TG) and cholesterol (CHOL) concentrations were determined by enzymatic colorimetric methods according to Boehringer Mannheim automated analysis for BM/Hitachi system 737 (July 1984 edition). Folate concentrations in serum were determined by no-boil dual count radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA).

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

Results

Distribution of 7-OH-MTX in-vitro. Distribution of 7-OH-MTX to cellular components of whole blood in the 18 subjects studied was generally low, with a C/P ratio of 0·03 ± 0·06 (mean ± s.d., range 0–0·27) at pH 7·51 ± 0·05 (mean ± s.d.) (Table 1). Statistical analysis showed no significant difference between the two groups with respect to C/P ratios, and there was no significant correlation between C/P ratios and concentrations of HSA, TP, TG, CHOL, AAG and FOL in serum. Neither were the C/P ratios correlated to the protein binding parameters (%B, %F and B/F) of 7-OH-MTX in serum.

Table 1. 7-Hydroxymethotrexate (7-OH-MTX) cell/plasma (C/P) ratios, per cent bound (B) 7-OH-MTX in serum, and serum concentrations of albumin (HSA), total protein (TP), triglycerides (TG), cholesterol (CHOL), α_1-acid glycoprotein (AAG), and folate (FOL) in samples from the 18 volunteers. Results are given as mean ± s.d., group I consisted of healthy volunteers, and group II consisted of volunteers who had recently undergone hip surgery.

Groups	C/P ratio	B %	HSA $g L^{-1}$	TP $g L^{-1}$	TG mM	CHOL mM	AAG $g L^{-1}$	FOL nM
I (n = 11)	0·03	90·4	45·4	74·4	1·2	5·6	0·6	17
	±0·08	±3·3	±3·2	±3·9	±0·9	±1·3	±0·1	±9
II (n = 7)	0·03	82·3	30·3	53·1	1·1	3·7	1·4	9
	±0·03	±4·0	±6·5	±12·5	±0·3	±0·9	±0·3	±2
I + II	0·03	87·3	39·5	66·1	1·2	4·8	0·9	14
(n = 18)	±0·06	±5·3	±8·9	±13·4	±0·7	±1·5	±0·5	±8

Serum protein binding of 7-OH-MTX in-vitro. 7-OH-MTX was highly bound in serum, with a mean binding in the 18 subjects of 87·3 ± 5·3% (mean ± s.d.) at pH 7·41 ± 0·07 (mean ± s.d.). In the healthy volunteers, who all had HSA concentrations within normal limits (45·4 ± 3·2 g L^{-1}, range 39·9–49·8 g L^{-1}), the binding (%B) was 90·4 ± 3·3% (mean ± s.d., n = 11), while the corresponding value for the surgical patients, with HSA concentrations of 30·3 ± 6·5 g L^{-1} (range 23·3–39·6 g L^{-1}), was 82·3 ± 4·0% (mean ± s.d., n = 7) (Table 1). The Wilcoxon rank-sum test showed a statistically significant difference between the two groups with respect to binding of 7-OH-MTX ($P < 0{\cdot}0014$). As shown in Fig. 1, the degree of binding of 7-OH-MTX as a whole was closely correlated to HSA concentrations ($y = 0{\cdot}43x + 70$, $r = 0{\cdot}72$, $P < 0{\cdot}0007$, n = 18). To a lesser degree, there was also a positive correlation between %B and TP ($y = 0{\cdot}23x + 72$, $r = 0{\cdot}58$, $P < 0{\cdot}012$) and between %B and CHOL ($y = 2{\cdot}4x + 76$, $r = 0{\cdot}66$, $P < 0{\cdot}03$), but both TP and CHOL were intercorrelated with HSA concentrations. No correlation ($P > 0{\cdot}05$) was evident between %B and AAG, TG or FOL in serum.

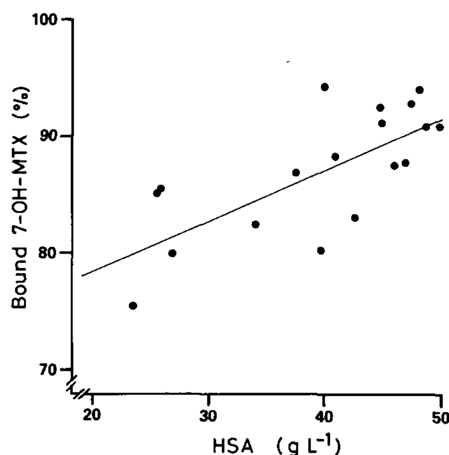

Fig. 1. Correlation between per cent bound 7-hydroxymethotrexate in serum and serum albumin (HSA) concentrations in samples from the 18 volunteers.

Discussion

For MTX, distribution data are available from in-vitro experiments in blood (Slørdal & Aarbakke 1987), investigations of distribution between plasma and peripheral lymph in adult patients (Sand et al 1978), and measurements of the

diffusion of the drug to cerebrospinal fluid in children (Evans et al 1983), as well as from experiments in rats (Miglioli et al 1985).

The in-vitro results presented herein show that after short-time incubation of 7-OH-MTX, only a tiny fraction of the compound is distributed to the cellular components in blood. Using radioactive drug, but otherwise similar experimental conditions, we have previously found a C/P blood distribution ratio of 0·3 for MTX (Slørdal & Aarbakke 1987), i.e. a ratio 10-fold higher than for the 7-hydroxylated metabolite. This is not inconsistent with the pharmacokinetic estimations of a 3-fold higher volume of distribution in the postdistributional phase observed for MTX compared with 7-OH-MTX (Slørdal et al 1986a). The lack of correlation between the calculated C/P ratios for 7-OH-MTX and binding parameters, concentrations of proteins, and folate in serum, probably reflects that the metabolite neither has binding sites on the red cell membrane, nor that it penetrates the membrane to any significant degree. However, it must be emphasized that our results are from short-time incubation experiments, which do not rule out equilibrium between unbound 7-OH-MTX in serum and intracellular drug, or, for instance, competition between 7-OH-MTX and endogenous folate for cellular entry, at later stages.

In man, therapeutic concentrations of MTX are 40–50% bound in serum (Paxton 1981, 1982; Breithaupt et al 1982), with HSA responsible for approximately 90% of the binding (Steele et al 1979). The only data available on the protein binding of 7-OH-MTX are from ultrafiltration experiments with serum reported by Breithaupt et al (1982), where the metabolite was stated to be 93 ± 2% bound, and from studies of 7-OH-MTX binding in HSA solutions, where 95% of 10^{-5} M 7-OH-MTX was bound (Lopez et al 1986).

The high degree of serum protein binding of 7-OH-MTX in healthy subjects (90·4 ± 3·3%, mean ± s.d.) is in agreement with data published previously (Breithaupt et al 1982). The positive correlation between bound 7-OH-MTX in serum and HSA concentrations (Fig. 1) further demonstrates that 7-OH-MTX in serum, like MTX, is mainly bound to HSA. The positive correlation between per cent bound 7-OH-MTX and cholesterol concentrations hardly reflects binding of 7-OH-MTX by lipoproteins, but may be explained by a convariation between the concentrations of HSA and cholesterol.

From a clinical point of view, the high degree of serum protein binding of 7-OH-MTX may be of importance. Although only approximately 10% of MTX administered during high-dose therapy is recovered in urine as 7-OH-MTX, plasma concentrations rapidly exceed those of MTX, and in the elimination phase, can be more than 10-fold higher than MTX concentrations (Breithaupt & Kuenzlen 1982; Winograd et al 1986; Slørdal et al 1986a). Because of an aqueous solubility 3 to 5-fold less than MTX, the 7-hydroxy metabolite has been proposed as a mediator of renal toxicity following high-dose MTX treatment (Jacobs et al 1976, 1977). 7-OH-MTX has further been demonstrated to inhibit influx, reduce polyglutamation, and enhance efflux of MTX in-vitro (Lankelma et al 1980; Fabre et al 1983a, b; Gaukroger & Wilson 1984; McGuire et al 1984). Depending on its distribution in man, 7-OH-MTX might thus modify target cell response and the clinical outcome of high-dose MTX therapy in several ways. The linear relationship between binding and HSA concentrations, as well as the significant reduction in bound fraction of 7-OH-MTX with low HSA concentrations, suggest that relatively high free fractions of 7-OH-MTX may be present in patients with hypoalbuminaemia, administered high-dose MTX therapy.

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