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APPEARANCE OF A METHOTREXATE BINDING PLASMA PROTEIN DURING HIGH DOSE METHOTREXATE THERAPY

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INTRODUCTION

Methotrexate (MTX) is one of the most widely used oncocytotopic drugs[1]. Recently it has been used in extremely high doses (8-15 gms/m^2) in the treatment of osteosarcoma[2], non-Hodgkin's lymphoma and metastatic head and neck cancer[3]. Such high doses facilitate the passive entry of MTX in most solid tumours and thereby provide the necessary tumour cell kill. However, to achieve the desired effects of this treatment, it is usually given repeatedly over extended periods of time depending upon the type of regimen being followed. Toxicity of the drug is prevented by ensuring its rapid clearance from the body by proper hydration, alkalinization and restoring to leucovorin "rescue"[4]. However, reports of delayed plasma clearance of the drug during the course of therapy in certain patients with osteosarcoma due to one reason or another and the ensuing toxicity are not very uncommon[5,6]. We report a patient suffering from osteosarcoma who developed severe toxicity following third infusion of MDC due to delayed plasma clearance of the drug.

CASE REPORT

A 19 year old male was admitted to The Aga Khan University Hospital with pain and tenderness in the left knee. Incisional biopsy from the lower end of the femur established the diagnosis of osteogenic sarcoma. Radiological examination of liver and chest indicated no evidence of metastasis. The patient was put on chemotherapy and was first given a course with cyclophosphamide- bleomycin-adriamycin and then two weeks later put on high dose MTX therapy (12 gm/m^2 every week). In every cycle of treatment, MTX (18.7 gm) was administered in the form of 6-hr infusion. This therapy was discontinued after the third MDC infusion as he developed symptoms of severe toxicity which included fever, chills, nausea, vomiting, maceration of skin, erythema, pancytopenia, etc. During chemotherapy, serum creatinine levels remained almost within the normal range. MIX levels in plasma were monitored by a very sensitive radioassay[7,8]. Leucovorin 'rescue' (15 mg/rn^2 every6hr) was started 24 hours post MIX infusion and continued till the levels of MTX dropped below 1×10^7 M.

Experimental methods

A. Immuno-precipitation of plasma proteins: In order to determine that the circulating MTX was bound to which type of plasma protein, 0.5 ml of patients plasma sample obtained 9 days following the second infusion of MDC was dialyzed extensively against normal saline at 4°C for 24 hours. Twenty five ul from the dialyzed plasma was used to set up each immune reaction. Three different immune reactions were set up each containing 25 ul of dialyzed plasma and 225 ul of 0.006M citrate buffer, pH 7.4. Then 250 ul of anti-human albumin or anti-human a1 acid glycoprotein or anti-human IgG was added to respective tubes. Appropriate control reactions were also set up. These contained 25 ul of normal saline with free MDC equal to the amount in the dialyzed patient's plasma, 250u1 of antibody to a plasma protein (anti-human albumin or anti-a1 acid glycoprotein or anti-human IgG) in a total volume of 0.5

ml made up with 0.006 M citrate buffer, pH 7.4. All reactions including controls were incubated at 4°C for 48 hours and then centrifuged at 3,000 rpm at 4°C for 15 min. The supernatant solutions were discarded and the precipitate in each tube was quickly washed with 0.5 ml of cold saline. The precipitate was then dispersed in 250/41 of 0.006M citrate, p11 7.4, and boiled for 5 mi to extract MDC which was then assayed by the radioassay[7,8].

Gel-filtration of patient's plasma

One ml of patient's plasma obtained 30 hours after the third MDC infusion was applied to a column (1.5 cmx75 cm) of sephadex G-75 as described earlier[9]. The sample was eluted with 0.05 M Tris-HCl buffer, pH 7.2 at a flow rate of 12 mI/hr. One ml fractions were collected and MIX in every other fraction was monitored by the radioassay. Dcxtran blue, human serum albumin and $3H_2o$ were used as column markers in a separate run on the gel.

Additional binding of [3H]MTX

Since most plasma proteins are non-specific binders of MDC additional binding of [H]MTX at pH 7.2 by patient's plasma was carried out according to the method described previously[10] with the difference that 50/41 plasma was used as binder instead of L1210 cytosol and incubation of the reactions was carried out for 1 hour at 37°C.

Serum electrophoresis

Electrophoresis of two serum samples of the patient before and after second MDC infusion was carried out according to Beckman Paragon Electrophoresis system SPE (Beckman Instruments Inc., Brea, CA). The agarose gel after the electrophoresis was dried into a film which was then scanned in a Beckman (Appraise) densitometer.
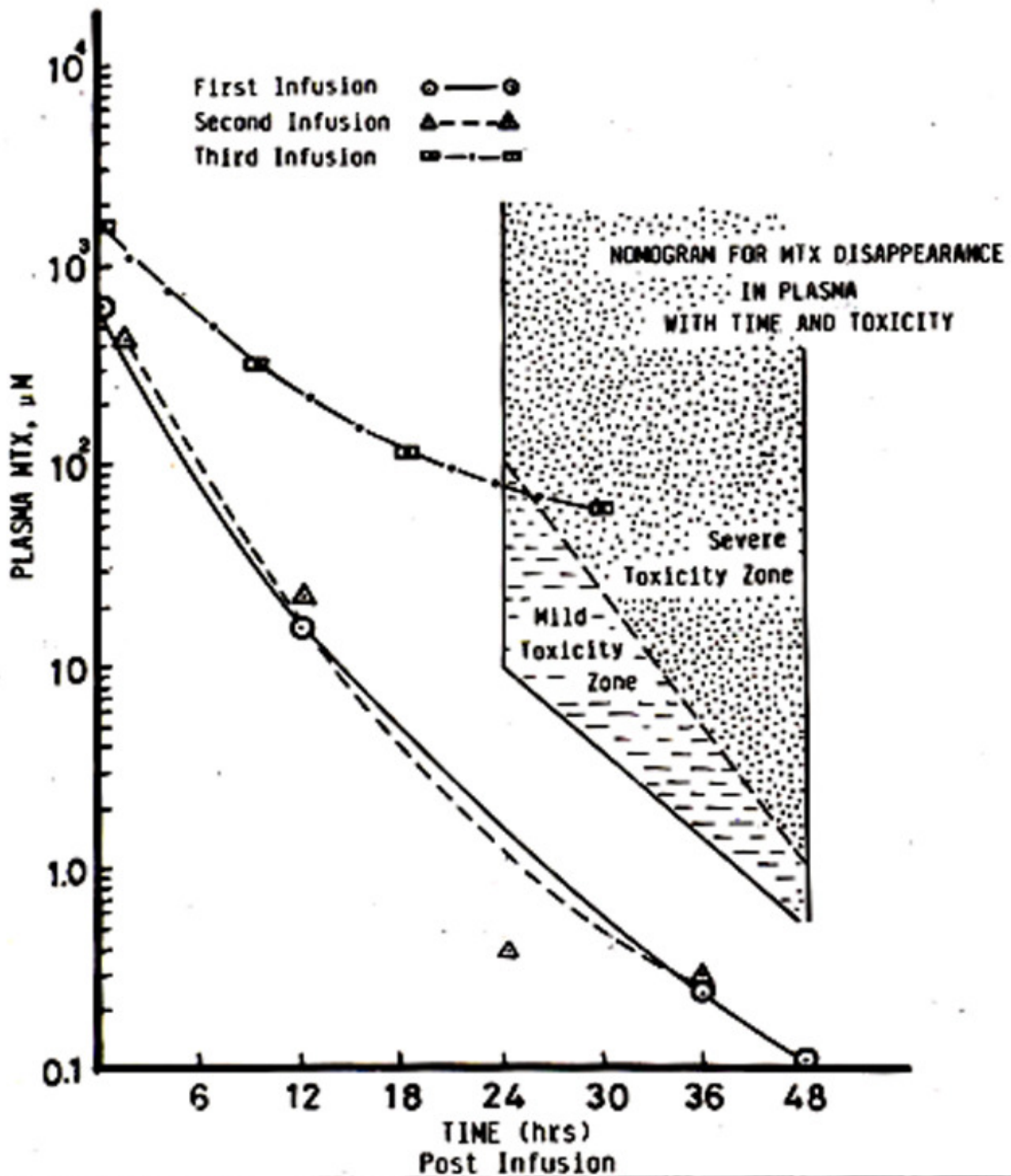

RESULTS AND DISCUSSION

Figure 1. Plasma concentration-time curves of MTX following 6-hr infusion of the drug (18.7 gm in each infusion). Nomogram for MTX disappearance in plasma with time and toxicity was constructed according to the references 16 and 17.

Figure 1 shows the decay curves of plasma MTX after 6 hr infusions (18.7 gms MTX in each infusion). The elimination of the drug during all three infusions appears to be biphasic during the first 48 hr. Initial phase of elimination half-lives (t1/2a) during the first and second infusions was calculated to be 1.5 hr - 2 hr while the second phase of elimination (t1/2B) was found to be approximately 5 hr. .6 hr.

These half-life values were not much different from those reported by us previously and by other investigators[11-13]. However, during the third infusion, there was a substantial change in elimination half-lives of the drug. The t1/2a value was 3.5 hr - 4 hr while t1/2B value turned was more than 12 hr since binding of a drug to plasma proteins has been known to significantly influence, its pharmacodynamic properties and pharmacokinetic parameters, this slower plasma clearance of MTX following third infusion was attributed to be due to its binding to a plasma protein[14,15]. This binding of the drug to the plasma protein must be strong enough so that it does not come off very easily. Different samples of the plasma taken at different time intervals following MDC infusions were analyzed for their ability to bind [3HJMTX.
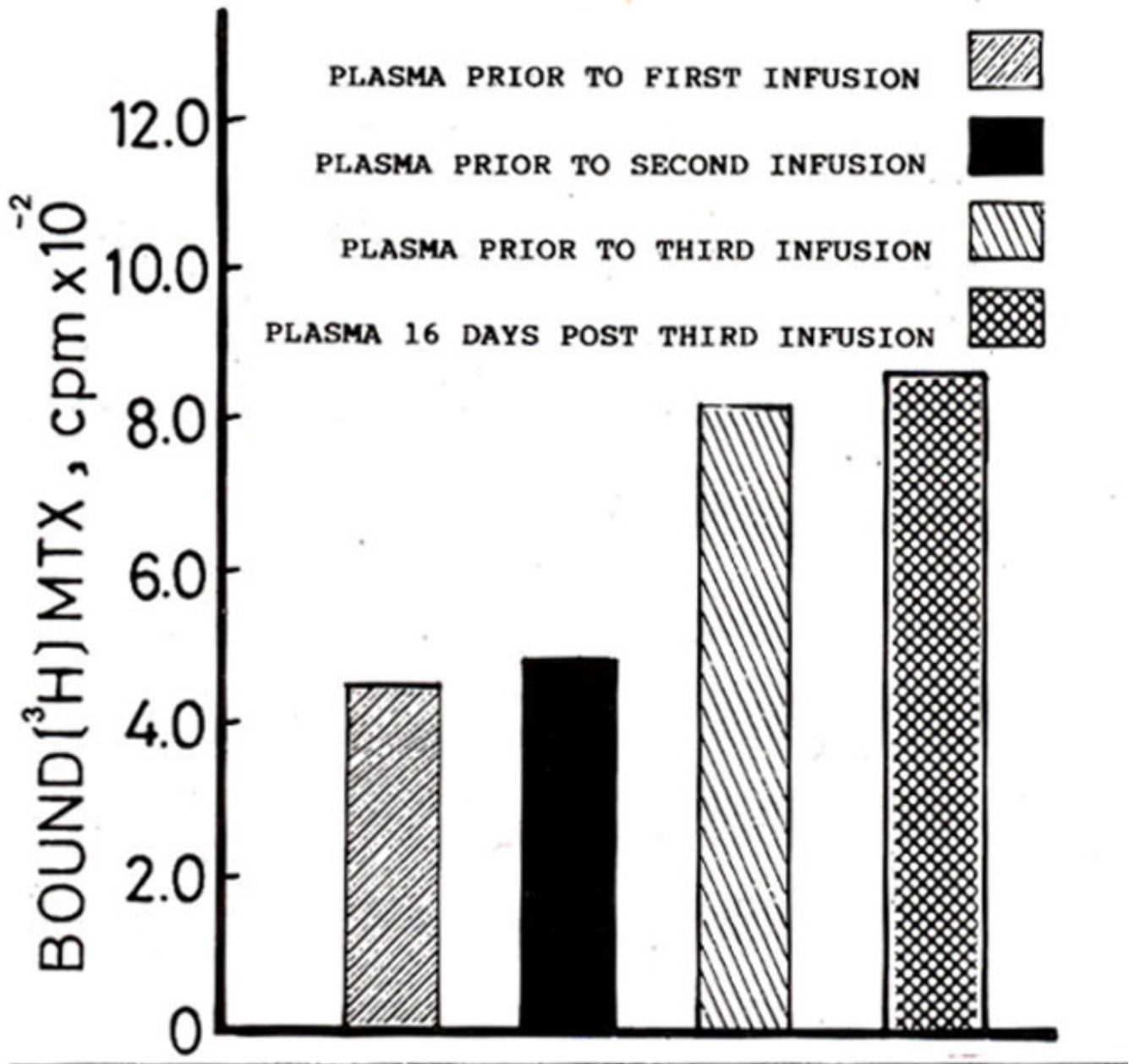

Figure 2. $[^3H]$MTX binding capacity of patient's plasma before and after different infusions of MTX.

Figure 2 shows additional binding of [3H]MTX by patient's plasma before and after first, second and third MDC infusions, it is evident that following second infusion, there appears something in plasma

that can bind [3]MTX more avidly and this binder of MDC persists in plasma even 16 days post third infusion. To find out the exact nature of this binder of MTX, patient's plasma obtained after second infusion was extensively dialyzed against normal saline and then treated with anti-human IgG, anti-human a1 acid glycoprotein and anti-human albumin in separate reactions.

TABLE. Methotrexate concentration in patient's plasma and in immune precipitates.

Sample	MTX concentration in plasma (ng/ml)		MTX concentration in immune precipitates (ng/ml)		
	Before dialysis	After dialysis	Anti-human IgG	Anti-human acid glycoprotein	Anti-human albumin
Patient's plasma post 2nd infusion	410	125	0	0	11.8
Control* (MTX in PBS)	125	-	0	0	0

*Control reaction was prepared by adding cold MTX in phosphate buffered saline (PBS) equal to the concentration in patient's plasma post 2nd infusion. The immune reactions using these two samples (patient's plasma and control) were run as described in Materials and Methods section.

Table shows that the bound MTX in patient's plasma was complexed with albumin. Further confirmation of this observation was obtained by subjecting patient's plasma (following third course of MTX therapy) to Sephadex G-75 gel- filtration chromatography (Figure 3).
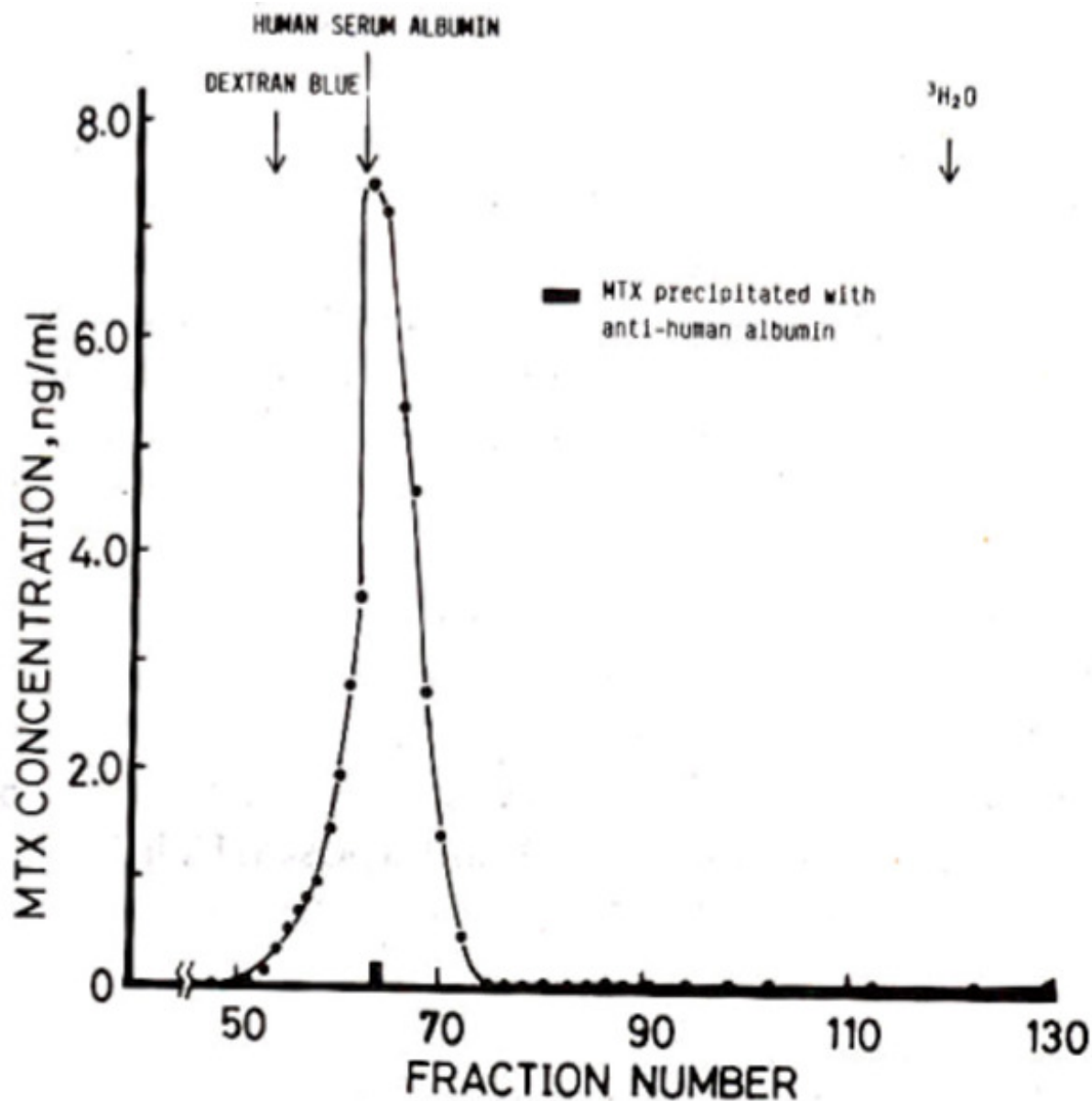
Figure 3, Gel filtration through Sephadex G-75 of patient's plasma following third course of MTX therapy. The solid bar on x-axis indicates MTX precipitated with antiserum to human albumin.

There is a single peak of MDC bound to a protein which appears to have a size nearly that of human serum albumin. Immunoprecipitation of 0.5 ml of fraction 65 (in the peak region) with anti-human albumin (1 ml) revealed MTX-albumin complexes in the peak (solid bar in Figure 3) lending further support to the notion that it is the albumin to which MDC has been bound in patient's serum. Non-specific binding of MDC to human serum albumin has been known for quite some time. However this binding is quite weak with two binding constants, 8.88 mM-1 and 17.6mM-1 respectively[15]. The binding of MDC to albumin in patient's plasma following second infusion is relatively stronger as a significant portion of the drug did not come off during dialysis of the sample (Table). Moreover, [3H]MTX bound to it did not come off with activated charcoal used in our radioassay (Figure 2). We are not sure whether this increased binding of MTX to patient's plasma is due to induction of albumin with high affinity for MDC or modification of a fraction of albumin to a high affinity form, however, serum proteins profiles of the patient's serum samples (before and after the second infusion) following
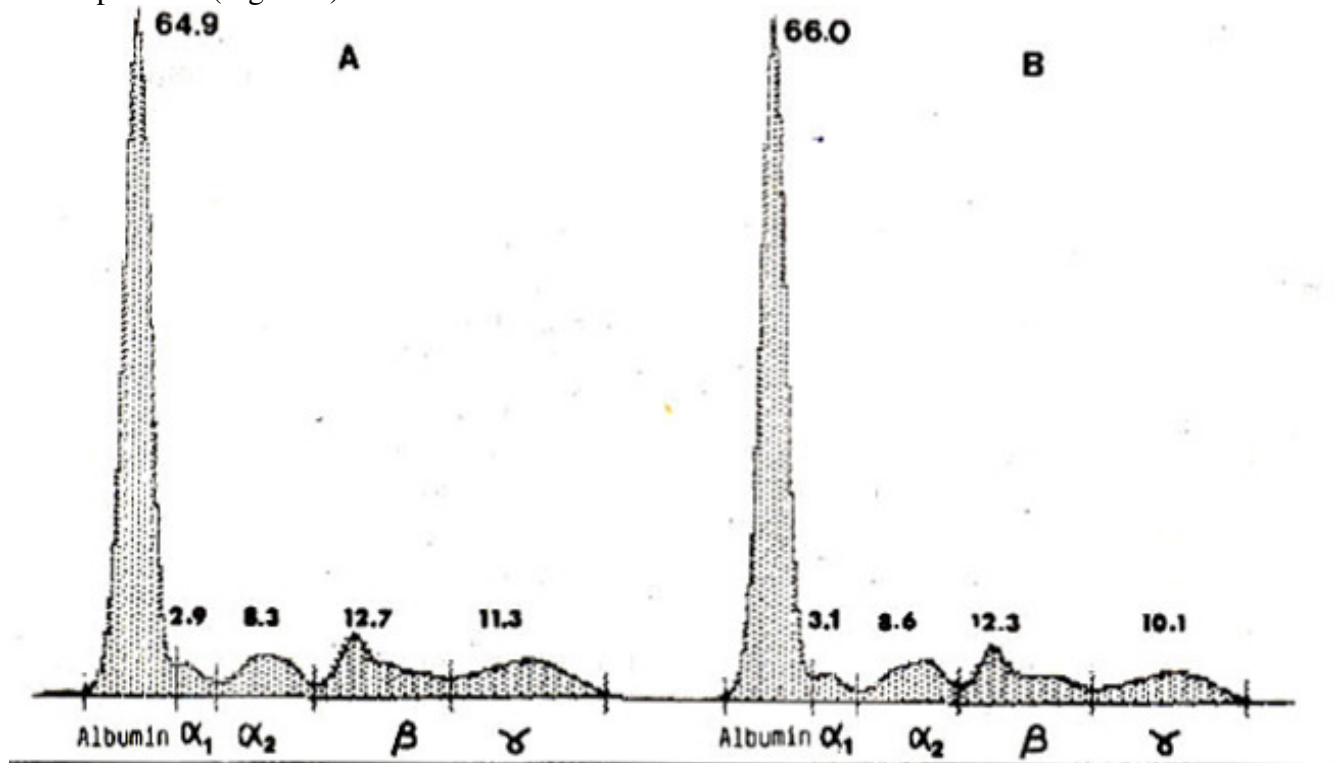
electrophoresis (Figure 4)

Figure 4. Serum proteins profile of patient's serum samples following electrophoresis. "A" refers to the serum sample before second infusion, while "B" refers to the serum sample after second infusion. Numbers on top of every peak are the relative % values of various serum fractions.

show that there is hardly any difference in the levels of albumin in these two samples. It is the property of a non-specific binder that it binds more of a drug at higher concentrations of the drug in the reaction. The binding of MDC to albumin before as well as after second infusion of the drug is of non-specific nature.
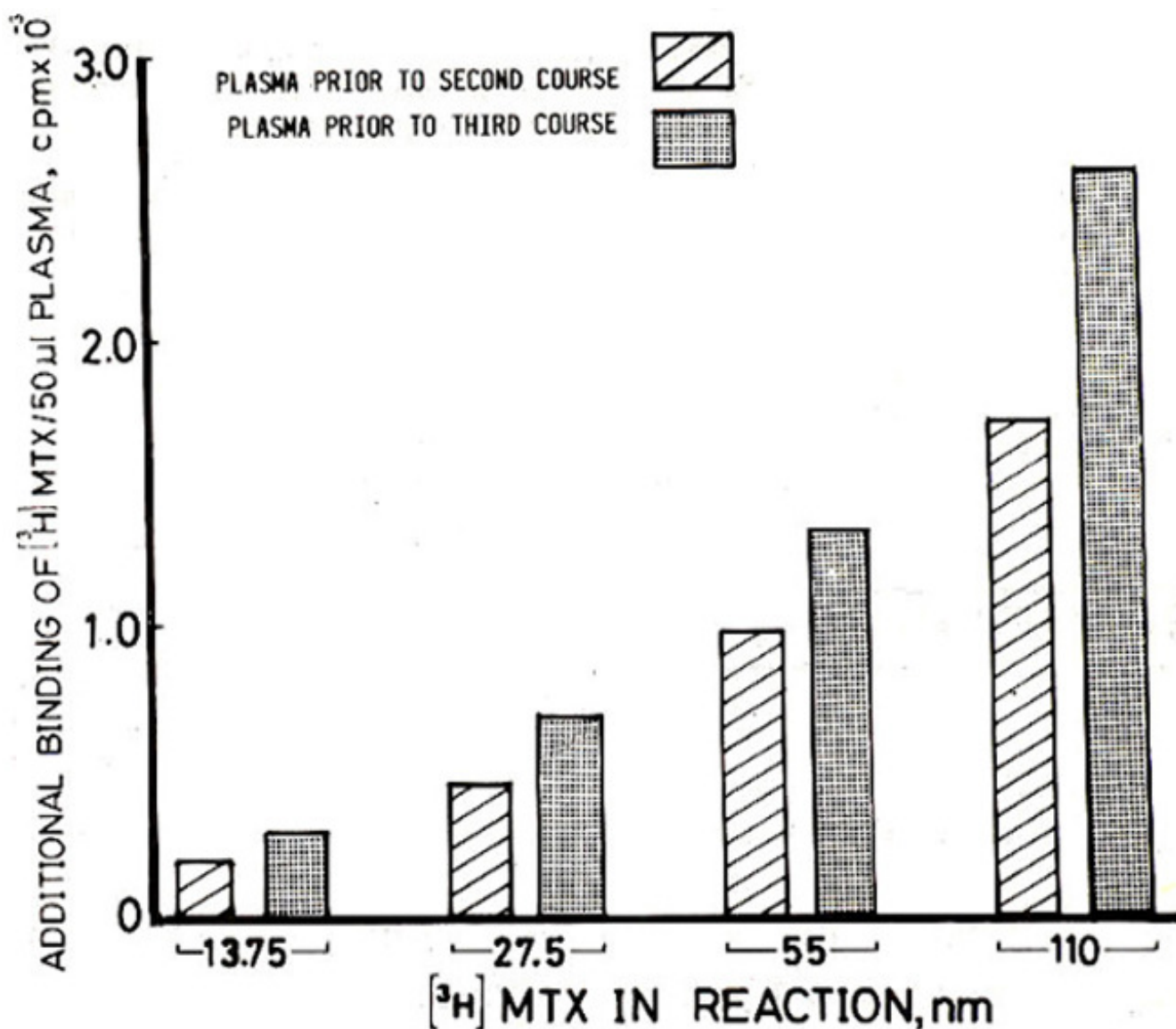
Figure 5. Additional binding of $[^3H]$MTX by patient's plasma prior to second and third course of treatment with MTX.

Figure 5 shows that I3H]IMTX binding by the two plasma samples progressively increases as MDC concentration in the reaction is increased from 13.75 nM-110nM. This indicates that albumin present in plasma before second infusion and after second infusion binds MDC non-specifically, though there is increased capacity of the second sample (after second infusion) to bind MDC and this perhaps is the reason for delayed plasma clearance of MDC in this patient In general, delayed clearance of the drug has been considered to be due to compromised renal function[13]. This report suggests that in certain patients during the course of treatment, certain plasma proteins may undergo a change leading to increased binding of MDC to plasma. This would delay the clearance of the drug resulting into severe clinical toxicity.

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