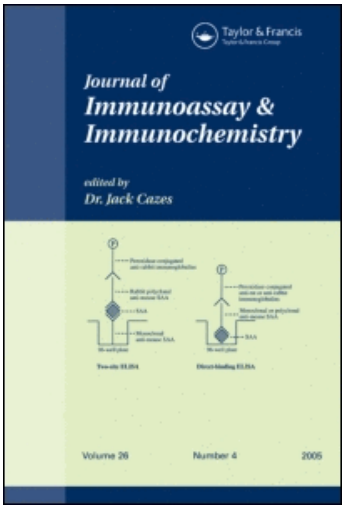

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A Radioimmunoassay for Methotrexate Adapted to the Centria System 2

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A RADIOIMMUNOASSAY FOR METHOTREXATE ADAPTED TO THE CENTRIA SYSTEM 2

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ABSTRACT

An automated radioimmunoassay for methotrexate using an iodinated tracer has been applied to the centrifugal analyser, Centria System 2.

Results obtained for serum samples correlated closely with those using a manual radioimmunoassay method. A major advantage of the assay is its potential for processing large numbers of samples rapidly, making it highly suitable for routine clinical use.

INTRODUCTION

The antifolate drug, methotrexate (MTX) is extensively used in the treatment of various forms of neoplastic disease. Both toxicity and effectiveness are related to serum levels of the drug and duration of exposure (1), and the monitoring of its concentration in the blood plays a vital role in the treatment of patients undergoing chemotherapy with high doses of the drug.

Existing methods for the measurement of MTX include microbiological (2), spectrophotofluorimetric (3), spectrophotometric enzyme inhibition procedures (4) and high pressure liquid chromatography (5). Radioimmunoassays (RIA) involving the use of tritiated MTX as tracer have been reported (6-11) and more recently RIA methods using ^{125}Iodine and ^{75}Selenium as tracer have also been introduced (12, 13, 14). Enzyme immunoassays have also been described (EMIT, Syva Corporation, Palo Alto, C.A. 94304; 15). Immunoassay techniques for the measurement of MTX are both extremely sensitive and easy to use, and are consequently being increasingly used in the clinical situation.

A radioimmunoassay for MTX using an ^{125}Iodine tracer applied to the Centria System 2 is described here.

MATERIALS AND METHODS

Reagents.

MTX, aminopterin, 4-amino-N^{10}-methyl pteroic acid and 2, 4-diamino-6-methyl pteridine were kindly supplied by Lederle Laboratories. Biologically prepared 7-hydroxy MTX was a generous gift from Dr. A.Jacobs, N.I.H., Bethesda. I^{125} sodium iodide (IMS 30) was obtained from the Radiochemical Centre, Amersham. Folic acid and its analogues, and Norit A Charcoal were purchased from Sigma Chemicals Limited; Dextran T-70 and Sephadex LH-20 from Pharmacia Limited; and isobutylchloroformate from Aldrich Chemicals. All other chemicals and solvents were obtained from BDH Chemicals Limited. Serum samples from patients receiving MTX were supplied by

Dr. G.P.Mould, St.Luke's Hospital, Guildford and Dr. H.E.M.Kay, The Royal Marsden Hospital, Sutton, Surrey. Union Carbide, France, provided the transfer discs, elution buffer and sheep double antibody tablets for use with their equipment.

Antibody.

The preparation of specific MTX antiserum has been described (11) and antiserum batch HP/S/3 IIIB was used in this study. The antiserum was used at an initial dilution of 1:20,000.

Radioligand.

^{125}Iodinated MTX was prepared following the method described by Kamel and Gardner (13) with the exception that a 50% methanol wash was added to the Sephadex LH-20 column at fraction 40 to elute the immunoreactive peak. The iodinated label was stable for approximately 6-8 weeks when stored undiluted at -20^{o}C. The label was diluted as required in assay buffer to give 20,000 cpm in 50µL.

Procedure.

The buffer used throughout the procedure was 0.05M phosphate, 0.1M NaCl, pH7.4, containing 2g/L BSA. Oxford^{R} dispensers or a Compu-pet^{R} (Warner Diagnostics Limited) were used for all dilutions of standards and patient samples. The amount of antiserum used was that dilution which bound 50% of the added label. The MTX standard was stored at 4^{o}C as a stock solution of 100 mg/L (2.20 x 10^{-4}mol/L) and was found to be stable for up to 2 months. This stock solution was used to prepare a working solution containing

10μg/L which was diluted to give a range of standards 0-8μg/L (0-1.76 x 10^{-8}mol/L).

The Centria System 2 consists of 3 sections: a pipettor/dilutor, and incubator/separator and a counter/computer. The pipettor/dilutor dispenses the standards or patient samples (50μL per well), antiserum (200μL per well) and tracer (50μL per well) into a transfer disc. The disc is then placed in the incubator/separator which mixes, incubates and separates the products of the reaction. Centrifugal force initiates all reactions simultaneously by moving reactants to the outer cavities of the disc for the first incubation (10 minutes) and then on to the columns for the second incubation (5 minutes). Separation of the free and bound products using double antibody tablets begins when the rotating disc accelerates to its second speed. The radioactivity remaining on the columns is counted in the counter/computer which counts 3 columns simultaneously and processes the data. The details of this procedure are outlined in Fig. 1.

Results obtained in the Centria were compared with those obtained by a manual RIA method (11) using ^{125}Iodine-labelled MTX (13).

RESULTS

Standard Curve.

Fig.2 shows the mean curve of ten consecutive MTX standard curves, set up on the same day, obtained using the Centria. Inter-assay variation of several standard curves set up on

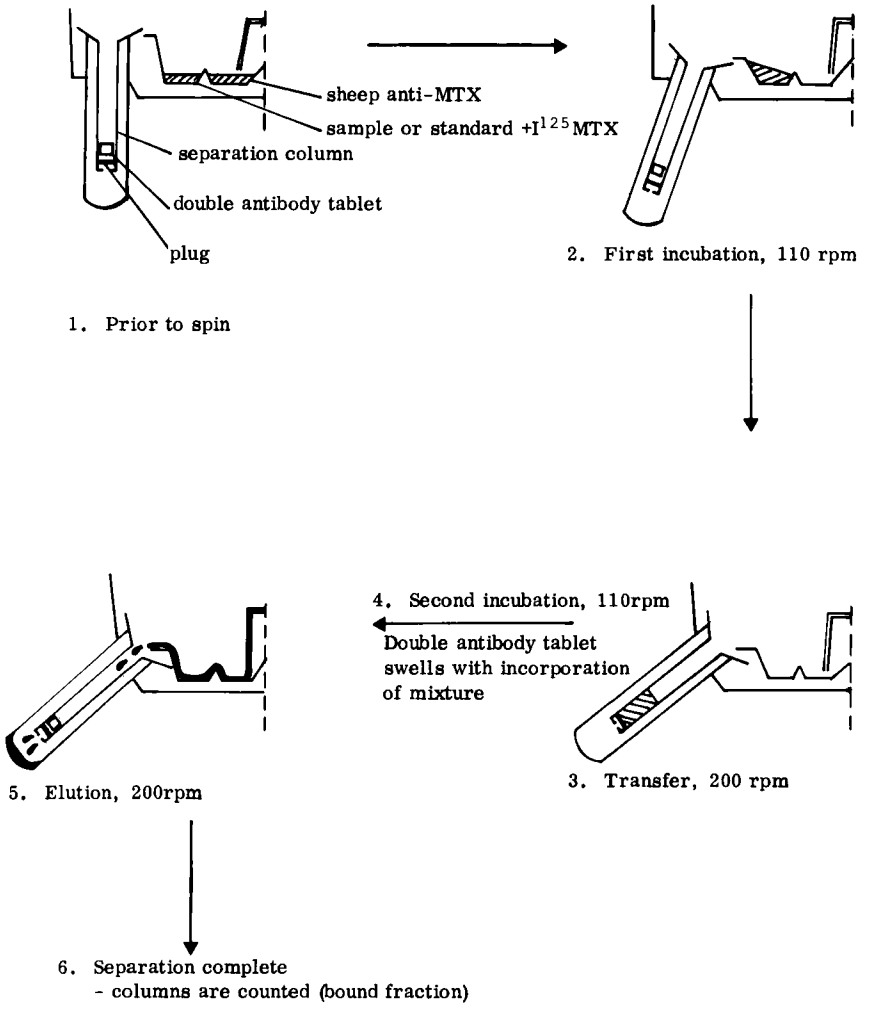

FIGURE 1 The Principle of the Centria System 2. Sheep anti-MTX (200µL), ^{125}Iodinated-MTX (50µL), samples or MTX standard (50µL). First incubation time (10 minutes), second incubation time (5 minutes), counting time per column (60 seconds).

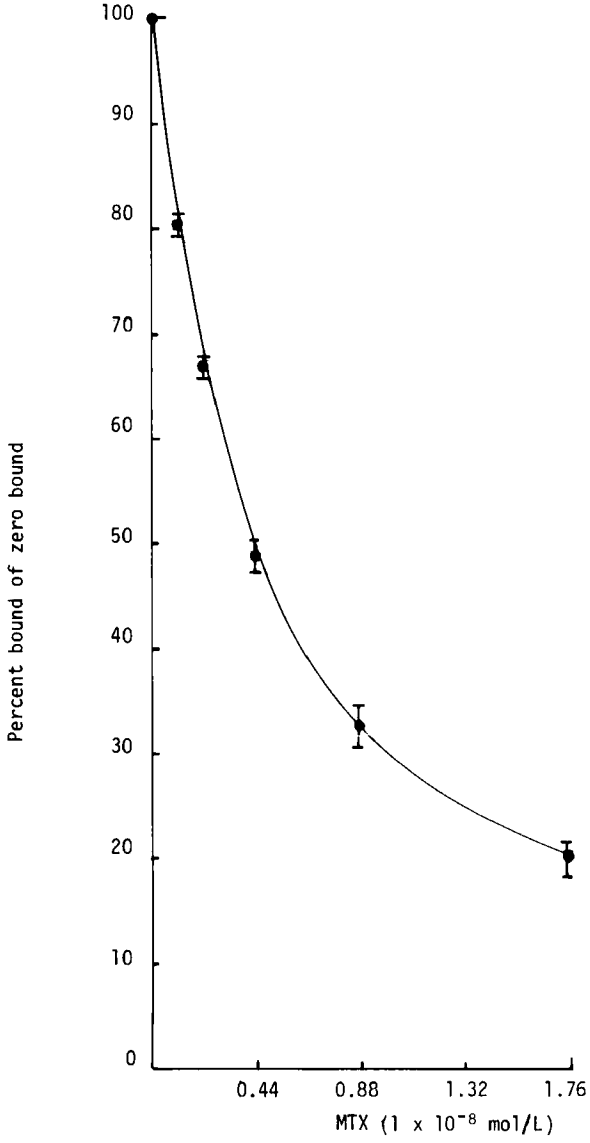

FIGURE 2 Intra-assay variation of 10 standard curves prepared using the Centria System 2. The mean and standard deviation at each point is shown.

consecutive days was also determined (Table 1). The sensitivity of the assay on the Centria was calculated to be 75ng/L (1.7×10^{-10}mol/L) (16).

The addition of 50µl of normal human serum to the standard curve did not significantly alter its shape or the percentage binding. MTX added to serum at a concentration of 100µg/L (2.20×10^{-7}mol/L) could be recovered quantitatively (96.8% recovery, n = 8) without prior treatment or extraction of the sample.

TABLE 1

DATA from 10 CONSECUTIVE STANDARD CURVES SET UP on the CENTRIA SYSTEM 2. MEAN and STANDARD DEVIATION VALUES ARE GIVEN FOR EACH POINT.

		Intra-assay Variation	Inter-assay Variation
zero binding (% total)		49.0 ± 1.5	47.4 ± 2.0
Standards (% zero)			
0.5	(0.11×10^{-8}mol/L)	80.4 ± 0.9	79.7 ± 1.9
1.0	(0.22×10^{-8}mol/L)	66.9 ± 1.0	66.1 ± 3.8
2.0	(0.44×10^{-8}mol/L)	48.9 ± 1.6	49.4 ± 4.1
4.0	(0.88×10^{-8}mol/L)	32.6 ± 1.8	32.9 ± 3.3
8.0µg/L	(1.76×10^{-8}mol/L)	20.2 ± 1.5	20.1 ± 2.9

Clinical Samples.

Two pools of sera from patients receiving MTX as part of their treatment were assayed several times on the Centria over a period of one month. Mean values of 0.84×10^{-6}mol/L (n = 15, coefficient of variance 10.0%) and 1.2×10^{-5}mol/L (n = 6, coefficient of variance 9.5%) were obtained. Intra-assay variation (C.V.) was 3.3% (n = 10, mean value 0.82×10^{-6}mol/L) and 3.0% (n = 10, mean value 1.14×10^{-5}mol/L) for the low and high quality control pools respectively.

The MTX concentration in 76 serum samples obtained from patients receiving MTX treatment was measured by both methods. The concentrations ranged from 0.88×10^{-9}mol/L to 5.73×10^{-4}mol/L. The correlation coefficient, r, was 0.998, $P < 0.001$, $y = -0.082 + 0.990x$ when y represents the results obtained on the Centria. The results are illustrated in Fig.3.

Specificity of MTX Antiserum.

Antiserum HP/S/3 IIIB was assessed for its cross-reactivity in both methods by replacing standard MTX in the assay with analogues of MTX at concentrations up to 2.20×10^{-4}mol/L. Results obtained with structurally related compounds are shown in Table 2.

DISCUSSION

The antiserum chosen for the development of a MTX RIA on the Centria was available in large quantities and had a high titre.

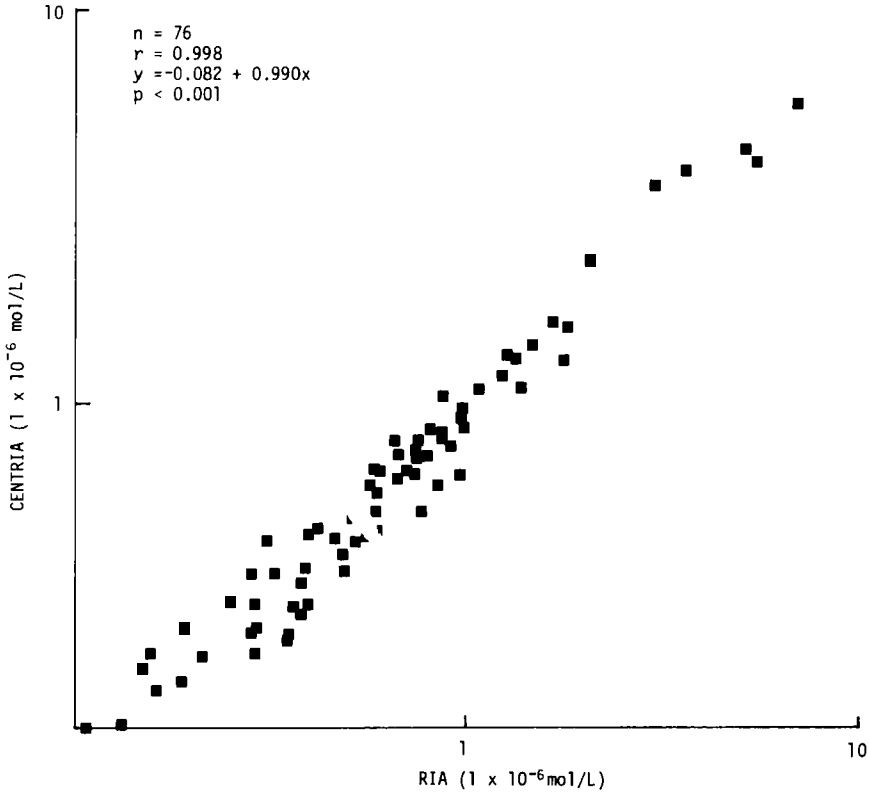

FIGURE 3 Correlation between serum MTX levels achieved using the Centria and Manual RIA methods.

This antiserum was obtained following one prime and three booster injections with a methotrexate-ovalbumin immunising conjugate and had similar specificity characteristics to an earlier bleed (HP/S/3 IIA) from the same animal (11). As with other published RIAs for MTX, the major cross-reactant was 4-amino-N^{10}-methyl pteroic acid, and the significance of any interference by this minor metabolite on MTX measurements in serum samples has still to be adequately defined.

TABLE 2

SPECIFICITY of ANTISERUM HP/S/3 IIIB USING BOTH THE CENTRIA and MANUAL RIA METHODS. THE CROSS-REACTION IS EXPRESSED AS THE RATIO of the AMOUNTS REQUIRED to PRODUCE 50% BINDING of ZERO.

	Centria	RIA
Methotrexate	100.0	100.0
4-amino-N^{10}-methyl pteroic acid	48.0	62.5
Aminopterin	55.0	39.3
7-hydroxy MTX	4.0	3.0
2, 4-Diamino-6-methyl pteridine	0.63	0.9
Folic Acid	< 0.009	0.5
Folinic Acid	< 0.009	0.005

Naturally occurring folates and folinic acid did not displace I^{125}-MTX bound to antibodies, even at concentrations 10^4 times greater than that of MTX, permitting the measurement of MTX to be made in the presence of artificially raised levels of folates (e.g. during folinic acid rescue).

The automated radioimmunoassay described in this paper has proved reliable and reproducible in routine use for monitoring MTX concentrations and has an inter-assay variation as low as 3.0%. The equation of the regression line shows a 16% difference between the two methods which could be due to the different separation

techniques used, these being dextran-coated charcoal and double antibody for the manual (11) and automated methods respectively.

Radioimmunoassay, especially when a gamma-emitting isotope is available, is, because of its speed and ease of performance, highly suitable for the clinical monitoring of patients receiving chemotherapy. The application of the MTX RIA to the Centria enables the operator to process a large number of samples a day as each run, with a maximum capacity for 18 samples, takes only 30 minutes to complete.

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