Assay of rifampicin in serum

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SYNOPSIS Two methods for the assay of rifampicin in serum are described. The first is a conventional plate diffusion method, measuring concentrations down to 0.02 µg/ml, and the second a chemical extraction followed by measurement of the inhibition of uptake of 14C-uridine by Staphylococcus aureus, which estimates in the range of 0.02 to 0.001 µg/ml. The methods were used to measure serum concentrations in man following doses of about 1050 mg and 75 mg rifampicin.

The use of rifampicin in intermittent regimens for the treatment of tuberculosis has the advantages of reducing the high cost of the drug and allowing full supervision of drug taking but is accompanied by side effects, probably immunological in origin (Poole, Stradling, and Worlledge, 1971; Aquinas, Allan, Horsfall, Jenkins, Wong, Girling, Tall, and Fox, 1972). Amongst various methods under investigation which might eliminate these side effects is the prescription of a small daily dose of rifampicin to be taken on the days between the larger therapeutic intermittent doses. Such an attempt to induce immunological tolerance is currently under study in Hong Kong using a daily dose of 75 mg rifampicin and in Singapore using 25 mg. The preliminary results of the study in Hong Kong suggest that administration of the small daily dose in between weekly doses of 900 to 1200 mg reduced the incidence of the ‘flu’ syndrome (Hong Kong Tuberculosis Treatment Services/British Medical Research Council, 1974). The serum concentrations attained were studied as they are fundamental to an interpretation of the results. Since the small daily doses used in the two studies were expected to produce concentrations of rifampicin only briefly measurable by conventional plate diffusion assays, a more sensitive method of assay was developed. In this method, serum proteins were precipitated with methanol and rifampicin was then extracted into chloroform and thence into water with the aid of n-heptane. The aqueous extract was evaporated to dryness and dissolved in nutrient broth seeded with Staphylococcus aureus. The rifampicin concentration could be measured as inhibition of the uptake of uridine-C14 into the staphylococcal RNA, since it inhibits bacterial deoxyribonucleic acid-dependent ribonucleic acid (RNA) polymerase (RNA nucleotidyl transferase E.C.2.7.7.6) (Hartmann, Honikel, Knüsel, and Nüesch, 1967; Wehrli, Knüsel, Schmid, and Staehelin, 1968; Sippel and Hartmann, 1968; Umezawa, Mizuno, Yamazaki, and Nitta, 1968). The present report describes a conventional plate assay which can measure down to 0.02 µg/ml rifampicin, the new method which can measure between 0.02 and 0.001 µg/ml, and the serum concentrations obtained with the large and the small doses of rifampicin in patients in the Hong Kong study.

METHODS

ASSAY ORGANISM Staphylococcus aureus (Staphylococcus subgroup I, NCTC 10702), resistant to streptomycin and other antibiotics (Mitchison, Allen, and Miller, 1970), was used as the test organism in both methods. Samples of an overnight culture in nutrient broth (Oxoid, no. 2) were stored at −196°C and an ampoule was thawed for each assay.

PLATE DIFFUSION ASSAY

Seeded assay medium was prepared by adding 10 µl culture to 100 ml Bacto antibiotic medium no. 1 (Difco, Detroit), modified by the addition of 3 ml M KH2 PO4, and was dispensed in 10-ml volumes into 9 cm diameter plates. Four wells, 8 mm diameter, were cut in each plate. Standards were made by adding rifampicin in two-fold dilutions to pooled human serum to make concentrations from 1.28 to 0.02 µg/ml. The standard and unknown sera were each pipetted to fill four wells in separate plates. After overnight incubation at 37°C, the diameters of the zones of inhibition (including the well) were

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measured with vernier calipers. A linear relationship was found between zone diameter and log rifampicin concentrations (fig 1). Sera containing more than 1.28 µg/ml rifampicin were diluted 1:40 in human serum and the test was repeated.

I N C O R P O R A T I O N O F U R I D I N E

Each tube containing dried extract received 0.4 ml of buffered medium pH 6.4, prepared by mixing 5 ml 0.5 M tris maleate buffer pH 6.6, 25 ml nutrient broth (Oxoid no. 2) and 0.6 ml 0.2 N HCl, and the contents were dissolved by agitating the tube on a Vortex mixer. To each tube was added 0.1 ml of a mixture of 4.5 ml nutrient broth, 0.3 ml of the culture of Staph. aureus, and 10 µCi in 0.2 ml 14C-uridine (uridine–2–14C, specific activity about 60 µCi/mM, Radiochemical Centre, Amersham). After incubation in a water bath for three hours at 37°C, incorporation was terminated by the addition of 5 ml 5% (w/v) trichloracetic acid. The organisms were collected on glass fibre paper discs (Whatman, grade GF/A) dried and counted in toluene containing 7 g/l 2(4-tert-butylphenyl) 5(4-biphenyl) 1-3-4 oxadiazole in an Intertechnique ABAC 40 scintillation counter. Serum without rifampicin yielded about 60 000 cpm and serum with an excess of rifampicin about 120 cpm.

C A L C U L A T I O N

A plot of log cpm against log rifampicin concentration was sigmoid. Approximate linearity was obtained over the range 1-20 ng/ml if the counts were transformed to log \( \frac{R}{1-R} \), where R is the ratio of the activity (cpm) of the test serum to the mean activity of the two sera without rifampicin (fig 2).

R U R I D I N E U T P A K E A S S A Y

Standards
Standards were prepared containing 20, 10, 5, 2.5, and 1.25 ng/ml rifampicin in normal human serum. One sample (2 ml) of each standard and two samples of serum without rifampicin were included in each batch of assays.

Extraction
Samples of 2 ml serum, in a glass stoppered tube, were mixed with 6 ml methanol to precipitate protein and strip off bound rifampicin. After centrifugation at 0°C, 6 ml of supernatant was removed to a fresh tube, to which was added 3 ml chloroform and 1.5 ml 15% ammonium sulphate solution. After mixing, 3 ml of the lower chloroform phase was transferred to another tube and 4 ml n-heptane added. The tube was gently mixed and two 0.35 ml portions from the lower aqueous phase were transferred to separate tubes. These tubes were placed under reduced pressure on a rotary film evaporator, modified to process five tubes simultaneously, until only a drop of moisture was visible. Drying was completed by storing the tubes overnight over CaCl₂ in a vacuum dessicator at 4°C.

Fig 1  Relationship between the diameter of the zone of inhibition and log rifampicin concentration obtained with standards in a typical plate diffusion assay.

Fig 2  Relationship between \( \log_{10} \left( \frac{R}{1-R} \right) \) and log rifampicin concentration obtained with standards in a typical uridine uptake assay. R is the ratio of the activity of a standard serum containing rifampicin to the mean activity of sera without rifampicin.
Assay of rifampicin in serum

Stability of rifampicin in serum
Samples of human serum containing known concentrations of rifampicin were stored at -20°C and assayed at intervals of up to 33 days. No change in the concentrations occurred.

Serum for standards
No alternative to human serum for the preparation of standards was found. Considerably less rifampicin was recovered from aqueous solutions than from serum, suggesting that substances in serum, which passed through the extraction procedure, were protecting against adsorption of rifampicin onto the glassware. Recovery from horse serum and 4% solutions of bovine or human albumin was higher than from water but considerably lower than from human serum. The curve obtained with fresh frozen human plasma was similar in shape to the curve with human serum, but insufficiently close to allow its use.

Glassware
To avoid transmission of adsorbed rifampicin from one experiment to the next, scrupulous washing of all glassware proved essential. Tubes were scrubbed in Pyroneg (Diversey Ltd, Cockfosters, Herts) solution, rinsed in tap water, left in N HCl overnight and rinsed with water, methanol, and acetone before re-use. Fresh pipettes, usually disposable, were used for each dilution. Failure to observe these precautions resulted in spuriously low counts in some tubes.

Patients
Blood was collected from 10 Chinese patients admitted to a study of reserve drugs for the treatment of pulmonary tuberculosis (Hong Kong Tuberculosis Treatment Services/British Medical Research Council, 1974). These patients received at weekly intervals a dose of rifampicin of about 23 mg/kg body weight (900 mg for patients of less than 40-9 kg, 1050 mg for 40-9-49-5 kg, and 1200 mg for 50 kg or more), 90 mg/kg ethambutol, and approximately 66 mg/kg pyrazinamide given at the same time. On each day between the weekly doses they were also given a daily supplement of either 75 mg rifampicin or of a matching placebo. Blood was collected from patients allocated to the rifampicin-containing supplement after they had received at least one month of chemotherapy. Samples from the same patients were taken at intervals over a 48-hour period following one of the high weekly doses, but omitting the supplement during the collection period, and over a 24-hour period after a dose of supplement on the sixth day after the high weekly dose. The timing of rifampicin dosage in relation to food was the same as in the main study. The high weekly doses were given one and a quarter hours after a light breakfast. The supplement was given in the morning before food, except that patient 9 had taken breakfast one and a half hours previously.

Results

Accuracy of assays
In the plate diffusion method, the 95% fiducial limits of estimates of rifampicin concentrations in individual sera varied from ± 20% to ± 25% when the estimate was near the middle of the range of the standard concentrations to ± 40-50% near the extremities of the standard concentration range. The 95% fiducial limits for the uridine uptake method were approximately ± 30% for values near the middle of the standard concentration range.

Relative activities of rifampicin and desacetyl rifampicin
Desacetyl rifampicin (DARMP) is the principal metabolite of rifampicin and retains part of its antibacterial activity (Maggi, Vigevani, and Pallanza, 1968). However, the relative activity of rifampicin and desacetyl rifampicin varies from organism to organism. A comparison was therefore made of their relative activities against the test Staph. aureus and against Mycobacterium tuberculosis. For the experiment with M. tuberculosis, 11 drug-sensitive strains were obtained before chemotherapy from patients in Singapore and Hong Kong and, with strain H37Rv, were tested by the linear diffusion method of Lloyd and Mitchison (1964). In experiments 1 and 2 concentrations of 0-2, 0-6, and 1·8 μg/ml rifampicin and 0-6, 1·8, and 5·4 μg/ml desacetyl rifampicin were prepared in sterile water or human serum and were assayed in triplicate on plates seeded with Staph. aureus (fig 3). A good fit was obtained to the lines relating log concentration to zone diameter for rifampicin and desacetyl rifampicin and these lines were parallel, indicating similar diffusion rates of the two compounds. Experiment 3 was conducted in a similar manner with M. tuberculosis. The lines for rifampicin and desacetyl rifampicin again appeared parallel, but there were significant though small departures from linearity (0·025 < P < 0·05), a finding that does not invalidate the estimation of relative activity. Staph. aureus required about 4·5 times as much desacetyl rifampicin as rifampicin to produce zones of the same size (table I). The corresponding estimate for M. tuberculosis was 2·2.

Comparison of plate diffusion and uridine uptake assays
Serum specimens from patients receiving rifampicin
Table I  Relative microbiological activities of RMP and DARMP

which had low concentrations of the antibiotic by the plate diffusion method were appropriately diluted in normal human serum and assayed by the uridine uptake method (table II). The two sets of estimates agreed well.

Fig 3  Estimation of the relative activity of rifampicin and desacetyl rifampicin against Staph. aureus in the diffusion assay. The lines show the relationships between the diameter of the zone of inhibition and log rifampicin or desacetyl rifampicin concentrations.

Table II  Comparison of plate diffusion and uridine uptake assays

Table III  Serum concentrations of rifampicin (µg/ml) after high dose
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4-7 hours, respectively, were calculated for the same patients over the period of eight to 12 and 12 to 24 hours after a dose of the supplement when serum rifampicin concentrations were similar to those found at 24-32 and 32-48 hours after the high dose. The estimates of half-lives during these comparable periods were significantly shorter for the supplement than for the high dose (p < 0.01 and p < 0.05 for the earlier and later periods, respectively). Variance analysis showed no evidence of variation in half-life from patient to patient.

Discussion

Staph. aureus was chosen for several reasons as the test organism in preference to Sarcina lutea used by many other workers. Experience with the strain obtained from the National Collection of Type Culture (Micrococcus subgroup 1 NCTC 8340) suggested that it contained variants with different degrees of sensitivity to rifampicin and desacetyl rifampicin. Furthermore, the edge of the zone inhibition was less sharp and a longer incubation period was necessary before readings of the zone of incubation could be made.

A comparison of the relative activities of rifampicin and its metabolite desacetyl rifampicin showed that M. tuberculosis was relatively more sensitive to the metabolite than Staph. aureus. Since serum from patients treated with rifampicin contains desacetyl rifampicin, assays with Staph. aureus will slightly underestimate the total activity against M. tuberculosis. Estimates of the relative activities of rifampicin and desacetyl rifampicin for S. lutea, Bacillus bovis, and M. tuberculosis have been made previously by Cannetti, Djurovic, Le Lirzin, Thibier, and Lepeuple, (1970). Their estimate for M. tuberculosis, obtained by the inherently less accurate method of serial two-fold dilution, was similar to our estimate of 2:2. For the reasons stated above, we could not confirm their estimate for S. lutea.

The uridine uptake method of assay is considerably more time-consuming and difficult to standardize than the plate assay. This method was developed for measuring concentrations following low doses of rifampicin whose use is being explored in the prevention of toxicity occurring during intermittent dosage of the drug and for other reasons. For most purposes, however, the plate assay, capable of measuring down to 0.02 μg/ml, is sufficiently sensitive since the drug is used mainly for the treatment of tuberculosis and the minimal inhibitory concentration for M. tuberculosis is about 0.3 μg/ml.

The disproportion between peak serum concentrations and dose size and the slower elimination of rifampicin demonstrated four to eight hours after giving the high dose to the Hong Kong patients is similar to the results previously obtained by Furesz, Scotti, Pallanza, and Marpelli (1967) in man and by Dickinson and Mitchison (1970) in the guinea pig. The slower elimination of the high doses of rifampicin is probably due to saturation of biliary excretion of the drug. However, the differences noted in the initial rates of elimination of the high dose and supplement do not fully account for the disproportion in the peak rifampicin concentrations found. It is therefore suggested that there must be a 'sink', possibly of hepatic origin, that binds a greater proportion of the supplement than of the high rifampicin dose. Subsequent slow release from this sink could explain the differences in the rates of elimination of the two doses occurring at similar serum concentrations. The slower elimination of rifampicin noted after both doses when the serum concentrations fell below 0.04 μg/ml could have been due to release of rifampicin from protein binding. The immunological implications of the measurements of the serum concentrations following the large

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Geometric mean: 0-023, 0-18, 0-32, 0-55, 0-13, 0-036, 0-0062

Table IV Serum concentrations of rifampicin (μg/ml) after 75 mg dose

1 No specimen obtained.

weekly dose and the smaller daily supplement are discussed elsewhere (Hong Kong Tuberculosis Treatment Services/British Medical Research Council, 1974).

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References


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