

ELISA inhibition technique for the demonstration of sulphones in body fluids

II. A new method to monitor leprosy patient compliance under field conditions*

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Summary An enzyme-linked immunosorbent assay (ELISA) for sulphones in urine is described. This method for monitoring dapsone (DDS) self-administration is compared with the quantitative determination of DDS/creatinine (D/C) ratios, currently used. Urine samples collected from 10 volunteers on 14 consecutive days after taking single doses of 100 mg DDS are studied, together with pretreatment samples and urine containing standard amounts of DDS. The lowest quantity of DDS still visually detectable by ELISA inhibition is 0.01 $\mu\text{g/ml}$ urine. All samples obtained up to 4–10 days after the 100 mg DDS doses are positive by this method, whereas the first negative D/C results occur on the third day. The method is especially apt to monitor substantial failure in DDS self-administration without using sophisticated instruments.

Introduction

A current method to monitor the self-administration of dapsone (DDS) by leprosy patients is based on colorimetric estimation of the DDS/creatinine (D/C) ratio in urine. The method discriminates between blank control urine samples and urine samples of patients on DDS doses as low as 25 mg per day. Also urine samples collected within 2 or 3 days of a single 200 mg DDS dose were classified positive by this technique. The method was especially advocated by Ellard and colleagues¹ for quantitative estimation of percentages of self-administered doses that are actually being taken by groups of out-patients. With this aim it has been applied successfully to leprosy patients in Africa and Asia.^{2–13}

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However, the method has several limitations:

- (1) Variations in D/C ratios between controls on the same supervised DDS dose are too large for conclusions to be drawn as to the number of missed doses of DDS that might have preceded an individual low D/C ratio.¹
- (2) The estimation of low D/C ratios is limited by other diazotizable substances causing apparent D/C values in blank control urines. These values vary within a population, and also between different populations.^{1,2,4}
- (3) Compliance estimation may be hindered by the presence in urine of sulphonamides that cross-react with DDS in the colorimetric estimation. Measurement at a second wavelength is needed to avoid misleading results.²
- (4) The method requires a spectrophotometer, a costly and vulnerable instrument, not readily available in rural leprosy endemic areas.

In view of these limitations it is understandable that the WHO Expert Committee on Leprosy in its fifth report¹⁴ asks for *simpler methods for analysing urine for dapsona content*. The increasing incidence of resistance of *Mycobacterium leprae* to DDS makes a convenient method for checking leprosy patient compliance urgently needed in order to distinguish between relapse due to non-compliance and that due to the emergence of sulphore-resistant *M. leprae*. Such a method is also of importance in attempts to improve DDS self-administration, since poor compliance is probably a factor promoting the emergence of sulphone resistance.

We have therefore developed an enzyme-linked immunosorbent assay (ELISA) for DDS in urine. It is an inhibition technique essentially corresponding to an idea of Engvall and Perlmann,¹⁵ first applied by Holmgren and Svennerholm in 1973¹⁶ for the detection of *Vibrio cholerae* antigen in sera. Preliminary experiments with urine samples from 44 Kenyan leprosy patients have shown the high sensitivity of the basic technique, using whole rabbit anti-DDS serum and enzyme conjugated anti-rabbit IgG serum.¹⁷ The main tool for the test in its present form is the sulphones specific antibody-enzyme conjugate described in our previous paper.¹⁸ This paper describes the method for detecting sulphones in urine, and compares its qualities with those of the D/C method.

Materials and methods

URINE SAMPLES

Each of 10 healthy volunteers took a single oral dose of 100 mg DDS. Urine samples were collected immediately before the DDS doses were taken and on 14 consecutive days thereafter, from each volunteer one 10 ml sample per day. Early morning urine was avoided. Half of each sample was preserved by the

addition of a few grains of thymol. To each of the other 5 ml portions 2.5 ml 2 N HCl was added.

REAGENTS FOR ELISA

The following reagents were prepared according to the prescriptions laid down in the previous paper:¹⁸ Bovine Serum Albumin-DDS conjugate (BSA-DDS), lyophilized in carbonate buffer of pH 9.6 in vials containing 20 μ g BSA-DDS each, stored at room temperature; sulphones specific enzyme-immunoglobulin conjugate (the E-Ig dialyzate of the previous paper), mixed in portions of 125 μ l dialyzate with 500 μ l normal horse serum and 50 μ l Tween 20, lyophilized and stored at +4°C; purified 5-aminosalicylic acid (5AS), lyophilized and stored at room temperature in portions of 10 mg per vial.

Other reagents used were thymol, Tween 20, 5% aqueous phenol red solution, diluted ammonia solution, HCl, NaOH, H₂O₂, and phosphate buffered saline (PBS), containing 1.72 g Na₂ HPO₄ · 12H₂O, 0.254 g KH₂ PO₄ and 8.5 g NaCl per l of water.

DEMONSTRATION OF SULPHONES BY ELISA

Before analysis each urine sample was roughly adjusted to pH 7 by the addition of a few drops of diluted ammonia solution or diluted HCl, using phenol red as indicator. The presence of DDS and its conjugates in urine samples was demonstrated by an ELISA inhibition technique. A vial with lyophilized buffered BSA-DDS was filled with 10 ml tap water, dissolving the conjugate. To each of the 96 wells of a microtiter tray 100 μ l of this solution was added. The tray was covered (e.g. with a second one) and left to incubate for 15 min at 56°C. Then the tray was emptied and washed 4 times with PBS containing 0.05% Tween 20 (PBS/Tween), each time leaving the fluid in the wells for 1 min. Before the next incubation 25 μ l PBS/ Tween was added to each well, followed by 25 μ l of a urine sample. A vial with lyophilized E-Ig (one portion as described above) was filled with 5 ml tap water, dissolving the conjugate. To each well of the tray 50 μ l of this solution was added. The tray was again covered and left to incubate for 15 min at 56°C. It was then emptied and washed 4 times with PBS/Tween as described above. Finally to each well 100 μ l substrate was added, prepared by dissolving a 10 mg lyophilized 5AS portion in 9 ml distilled water, and then mixing it with 1 ml of a 0.05% solution of H₂O₂ in distilled water. After leaving the tray for 2 h at room temperature, the colour intensities resulting from the test samples were compared with those resulting from the pretreatment samples, and from a mixture of the latter to which standard amounts of DDS were added. Comparison was made with the naked eye.

ESTIMATION OF D/C RATIOS BY SPECTROPHOTOMETRY

DDS/creatinine ratios of the acidified urine specimens were estimated as described by Ellard and colleagues.¹

Results

Figure 1 is a photograph of ELISA results using the urine samples from 5 of the 10 volunteers. The first 2 rows show the results after using standard amounts of DDS in urine. The lowest quantity of DDS still detectable in these standard samples by naked eye is 0.01 $\mu\text{g/ml}$ urine, i.e. 0.25 ng/well (wells E 1–2). Likewise, in urine specimens collected up to 8 days after the 100 mg dose of DDS the naked eye could easily detect sulphones by ELISA. In the case of one volunteer even the urine collected on the tenth day was unmistakably positive (well C8). Similar results were obtained with the other urine samples, except in those of one volunteer, when the limit of detection was reached already on the fourth day after the DDS dose was taken. DDS/creatinine determinations proved that this particular volunteer was an extremely rapid DDS eliminator with an estimated $T_{\frac{1}{2}}$ (half-life) for DDS of 11 h, compared to the average $T_{\frac{1}{2}}$ among this group of volunteers of 22 h (range 11–34 h).

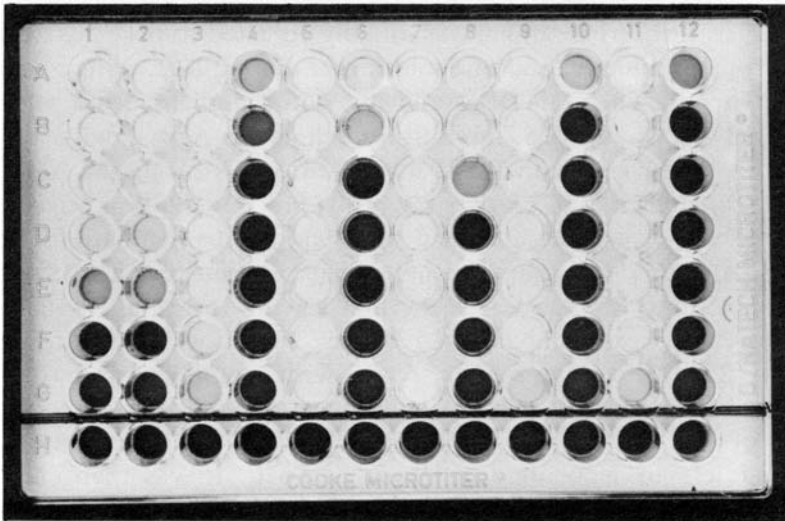


Figure 1. ELISA on urine samples to detect sulphones. Rows 1 and 2: duplicate mixed urine samples containing standard amounts of DDS, respectively 100 (A), 10 (B), 1 (C), 0.1 (D), 0.01 (E), 0.001 (F), 0.0001 (G) and O (H) $\mu\text{g/ml}$. Rows 3, 5, 7, 9 and 11: urine samples from 5 different volunteers, collected respectively before treatment (H), or on day 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F) and 7 (G) after taking 100 mg DDS. Rows 4, 6, 8, 10 and 12: urine samples from the same 5 volunteers, collected respectively before treatment (H, duplicate), or on day 8 (A), 9 (B), 10 (C), 11 (D), 12 (E), 13 (F) and 14 (G) after taking 100 mg DDS.

Table 1 compares the numbers of urine samples positive by D/C ratios with the numbers positive by ELISA. To obtain an end-point for the positive classification by D/C ratios either 2 or 3 standard deviations (0.7) were added to the mean pretreatment value (2.8), resulting in an end-point of 4.2 and 4.9 respectively. Using an end-point of 4.2, all samples collected up to 3 days after the 100 mg dose of DDS are positive by D/C ratio, but among those samples collected during the last days of the experiment some false positives are likely to have occurred. With an end-point of 4.9, only 7 out of 10 samples are positive on the third day, but false positives are excluded. The much greater sensitivity of the ELISA is apparent from the table. When $T\frac{1}{2}$ values of DDS are normal, urines may be expected to be positive for at least a week after the taking of a single dose of 100 mg DDS.

The ELISA results were identical, whether incubations were done for 15 min at 56°C, for 30 min at 37°C, or for 90 min at 20°C.

Table 1. D/C and ELISA analysis of urine samples after single oral doses of 100 mg DDS (10 subjects)

Time (Days)	D/C ratios ($\mu\text{g/ml}$)*	Number D/C > 4.9 [†]	Number D/C > 4.2 [‡]	Number pos. by ELISA
1	22.5 (15.2–27.8)	10	10	10
2	10.4 (6.5–14.3)	10	10	10
3	6.8 (4.3–10.3)	7	10	10
4	4.5 (2.7– 7.4)	3	5	10
5	4.1 (2.5– 6.4)	3	4	9
6	3.2 (2.1– 5.2)	1	2	9
7	3.0 (1.7– 4.3)	0	1	9
8	2.9 (2.1– 4.7)	0	1	9
9	2.7 (1.5– 3.8)	0	0	3
10	2.5 (1.5– 3.6)	0	0	1
11	2.4 (0.9– 3.2)	0	0	0
12	2.5 (0.4– 4.6)	0	1	0
13	2.7 (2.0– 4.4)	0	1	0
14	2.5 (0.8– 4.7)	0	1	0

* means and ranges

[†] mean of pretreatment values (2.8) plus three standard deviations (0.7)

[‡] mean of pretreatment values plus two standard deviations

Discussion

An important advantage of the ELISA compared to the D/C method is its higher sensitivity, even though it is read by naked eye. Reliable, positive results are obtained for up to 4–10 days after giving a single dose of 100 mg DDS, equivalent in effect to the situation at the end of a period of daily dosage with 50 mg DDS. Thus, finding negative results from urine samples of patients who

were meant to be self-administering such daily doses would indicate substantial failure in self-medication. This is clinically far more important than the occasional missed dose, and cannot be revealed with precision by the less sensitive D/C method.

Another advantage of the ELISA is its relative simplicity. No sophisticated instruments are required. A single microtiter tray will suffice for duplicate samples of 36 test specimens, 6 positive and 6 blank controls. For positive controls one might either use urine specimens containing a range of known DDS concentrations, as in this study, or serial dilutions of a mixture of urine samples from a small number of patients, whose DDS administration is strictly supervised. The latter will be easier to prepare in the field. It will also allow a more adequate comparison between control and test samples since both will contain a natural mixture of conjugated and unconjugated sulphones, each with its own affinity for the E-Ig conjugate.¹⁸

The turning-point of colour change should be included in the series of dilutions of the control urine. If that is done, calculations based on a $T_{\frac{1}{2}}$ of 1 day will give an indication of what the average negative urine result implies. It should be stressed, however, that the broad variations in diuresis and in $T_{\frac{1}{2}}$ for DDS do not allow any conclusions to be drawn as regards individual patients in anything more than general terms such as *substantial non-compliance*. Even quantitative tests, that include the creatinine determination as a correction factor for the diuresis, can avoid this limitation only by the laborious way of comparing individual results with those obtained after the same patient had taken supervised doses of DDS.^{1,2} Those who are interested in the omission of only 2 or 3 daily DDS doses should make different dilutions of the test samples, to find out at which dilutions a positive sample turns negative. Conclusions must again be based on average $T_{\frac{1}{2}}$ values, with the unavoidable restriction that a slow eliminator remains longer positive than a rapid eliminator, all other factors being equal.

For some leprosy workers such semi-quantitative interpretations of the ELISA may appear unnecessary or too troublesome. They want a simple *yes* or *no* answer to the compliance question. In this case, it may only be necessary to include 6 positive and 6 negative control urine samples, all undiluted. Any negative test result points to substantial non-compliance without further interpretation. Strict standardization of the reagents, giving reproducible tests under changing circumstances, does permit this simplification, thus making the test more attractive for the man in the field.

Although in the laboratory it was convenient to carry out our incubations at 56°C, clearly in the field incubations would be best done for 90 minutes at ambient temperature.

The shelf-life of two of the three lyophilized reagents is very long. They remained stable even when being kept at 56°C for one month. The third reagent, the specific E-Ig conjugate, remains in optimal condition kept at 4°C for one year, and at 37°C for 25 days.

The total production costs of the three lyophilized reagents per microtiter tray are about US \$15 (E-Ig, \$13; BSA-DDS, \$1; 5AS, \$1). These figures are an indication of the present situation. Other costs are the tray (\$0.50), the washing fluid and some chemicals (\$0.50 per tray). The trays may be recycled by intensive washing with 1% sodium dodecyl sulphate (SDS) and water, or with 1 N NaOH and carbonate buffer. If the investment of US \$100 for an adjustable pipette is prohibitive, simple plastic pipettes (\$0.25 each), calibrated for drops of 25 or 50 μ l of water and operated with a small balloon may be used.

In conclusion, this ELISA inhibition technique may be of some help to workers supervising leprosy treatment in endemic areas, especially where a growing resistance of *M. leprae* to sulphones occurs.

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