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ELISA inhibition technique for the demonstration of sulphones in body fluids

The use of dried blood on filter paper to monitor leprosy patient compliance*

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Summary Two enzyme-linked immunosorbent assays (ELISA) for sulphones in body fluids were adapted to measure sulphones in blood dried on filter paper. The more sensitive modification, based on competition between dapsone (DDS) and enzyme–dapsone conjugate, detects sulphone in blood extracts up till 6 days following 100 mg DDS intake. Application to monitor patient compliance is demonstrated, using finger-prick blood from 30 Ethiopian leprosy patients. Results are compared to those in urine, and to statements as regards the last daily dose of 100 mg DDS. Eight negative results were found, and employing serial dilutions of positive controls, this indicated that omissions of more than 5 doses in succession occurred. Practical aspects of the technique are discussed.

Introduction

In previous papers[1–2] we introduced an enzyme-linked immunosorbent assay (ELISA) on urines from leprosy patients, to monitor self-medication with dapsone (DDS). The test proved its potential value in Nigeria and Cameroon as a simple and sensitive tool for laboratories using less sophisticated equipment (unpublished data).

However, simple qualitative urine tests are inevitably influenced by diuresis.[3]

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Moreover, in some areas female patients often feel shy to produce a urine sample on request of the leprosy officer. We therefore considered the use of minute quantities of blood as an alternative to urine. It would simplify procedures for field work, if such samples could be collected as spots on filter paper.[4–5] In preliminary experiments it was found that DDS concentrations in extracts from dried blood spots obtained from volunteers taking standard DDS doses were just high enough to inhibit the ELISA presently used on urine (unpublished results). Apparently, the blood spot ELISA worked, but it was feared that under changing circumstances and in the hands of less experienced workers the detection limit for DDS might be too critical for practical work. Moreover, substantial failure in DDS self-administration would not be distinguished from the occasionally missed dose by this ELISA. Recently, the feasibility of the idea was enhanced by the development of an even more sensitive modification of the ELISA for sulphones.[6]

The present paper firstly describes the possibilities of the two ELISA's to demonstrate sulphones in dried blood samples from volunteers. Then, an application of the most sensitive test is described, using blood spots on filter paper obtained from leprosy patients in Ethiopia.

Materials and methods

BLOOD SAMPLES FROM VOLUNTEERS

Each of two healthy volunteers took a single dose of 100 mg DDS. Blood was taken by finger prick (4 x 50 μl) and by venapuncture (5 ml in a heparinized tube, and 5 ml in a tube without heparin), before and 1, 2, 3, 4, 5 and 6 days after the DDS intake. The finger-prick blood was collected in 50 μl heparinized capillary tubes that were emptied on small areas (1.5 cm diameter) of filter paper (Whatman 3). From each venous blood sample four 50 μl aliquots were immediately pipetted (Finn pipette) on similar small areas of filter paper. All filter paper samples were dried in air and stored at room temperature until analysed.

SAMPLES FROM PATIENTS AND CONTROLS

One 50 μl blood sample was collected from each of 30 outpatients of the All Africa Leprosy and Rehabilitation Training Centre (ALERT) at Addis Ababa. They belonged to a group of patients who received special attention for a variety of reasons such as suspected non-compliance with self-medication. The samples were taken when they visited ALERT periodically to collect 4 weekly supplies of 100 mg DDS tablets for daily self-administration. The blood was collected from finger pricks using 50 μl heparinized capillary test tubes, that

were emptied on small areas of filter paper. The papers were dried in air and stored at room temperature. Also, urine samples were collected from the patients and 5 ml aliquots were stored at room temperature after addition of a few grains of thymol. The patients were questioned about the last date of 100 mg DDS intake.

Positive control samples were collected from 6 inpatients of ALERT who took their daily doses of 100 mg DDS under strict supervision. The samples were collected just before a new dose was due to be taken. Blank control samples were collected from each of 6 healthy volunteers who were not taking DDS.

FILTER PAPER EXTRACTION

Each dried blood spot was cut out, slightly folded and dropped into a test tube (1.5 cm diameter). To each tube 500 μl phosphate buffered saline containing 0.05% Tween 20 (PBS/Tween) was added. The tubes were covered with parafilm and left overnight at 4°C. They were slightly shaken before use.

E-Ig BASED ELISA INHIBITION TECHNIQUE

This ELISA was done with freshly prepared sulphones specific E-Ig as described in the previous paper,[6] replacing the 50 μl aliquots of specified solutions of sulphones or analogues by 50 μl aliquots of filter paper blood extracts, and using an E-Ig dilution factor of 7×10^{-3}. The urine samples, however, were tested with the lyophilized ELISA reagents as previously described, unpublished data.[2]

E-DDS BASED ELISA INHIBITION TECHNIQUE

This ELISA also was done as described in the previous paper,[6] replacing the 50 μl aliquots of specified solutions of sulphones or analogues by 25 μl aliquots or normal horse serum containing 0.05% Tween 20 plus 25 μl aliquots of filter paper blood extracts, and using an E-DDS solution factor of 1.7×10^{-3}.

Results

The possible use of the two ELISA's to detect sulphones in different types of filter paper blood extracts was tested on blood samples from two volunteers who took single oral doses of 100 mg DDS. Figure 1 clearly shows that the E-DDS based technique is the most sensitive, which agrees with findings in the previous paper.[6] For an analysis of the results, the multi-scanner ELISA readings are listed in Table 1. The data may be summarized as follows:

1. The E-Ig based ELISA is 90–100% inhibited by filter paper blood extracts obtained 1 day after a single dose of 100 mg DDS, and it is about 50–60%

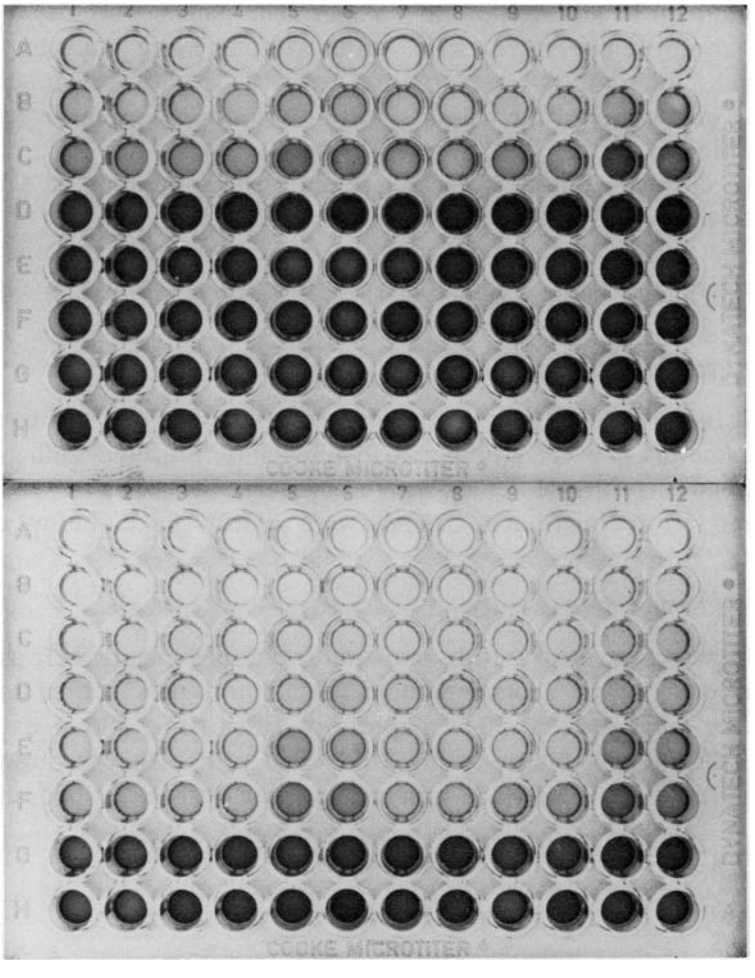

Figure 1. Two ELISA's applied to dried blood extracts from two volunteers who took single doses of 100 mg DDS. Top: E-Ig based ELISA; bottom: E-DDS based ELISA. Even rows are duplicates of foregoing odd rows.

Methods of blood collection: by venapuncture in a tube without heparin (rows 1, 2, 7, 8), by venapuncture in a heparinized tube (rows 3, 4, 9, 10), or by finger prick in a heparinized capillary tube (5, 6, 11, 12).

Times of blood collection: 1(A), 2(B), 3(C), 4(D), 5(E) and 6(F) days after 100 mg DDS doses, and before the DDS intake (G). Lines H show ELISA's on the extraction buffer for control.

inhibited by such extracts obtained 3 days after that dose.

2. The E-DDS based ELISA is 100% inhibited by filter paper blood extracts obtained 1, 2 or even 3 days after a single dose of 100 mg DDS, and it is about 50–60% inhibited by such extracts obtained 5 or even 6 days after that dose.

Table 1. Multi-scanner readings of two ELISA's applied to dried blood extracts from two volunteers who took single doses of 100 mg DDS

ELISA (basis)	Time (days)	Volunteer A			Volunteer B		
		VB*	VBH*	FPH*	VB	VBH	FPH
E-Ig	1	½	0	1	0	0	1
	2	1	1	2	1	1½	2½
	3	3	3	4	2½	3	5½
	4	7	7½	8	8	8	8
	5	9	9	8	9	9	9
	6	9	9	9	9	9	9
E-DDS	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	1	0	0	1
	4	1	1	2	1	1	2
	5	3	3	4½	3	3	4
	6	4	4	6	4	4½	6

The numbers are averages of duplicate results. The scanner was set on the optical densities resulting from the blank samples (Figure 1, lines G and H), thus printing these with the matrix numbers 8 or 9.

*VB = venous blood without heparin; VBH = venous blood with heparin; FPH = finger-prick blood with heparin.

3. Heparin does not influence the ELISA's, but the finger-prick blood samples apparently contain less sulphones than the samples obtained by venapuncture. However, this difference in contents is less than that found between samples obtained on two successive days from the same subject.

These results correspond with preliminary experiments on blood spots obtained from 6 volunteers up to 10 days after taking single doses of 100 mg DDS (unpublished data).

Because of its greater sensitivity, the E-DDS based ELISA was chosen for testing blood spots obtained from 30 Ethiopian leprosy patients. The control samples from the 6 inpatients who took their daily doses of 100 mg DDS under strict supervision, were initially analysed individually and undiluted, and subsequently in serial twofold dilutions after pooling of the extracts. Comparison of test samples with this type of control series gives an indication of what the average negative or positive result implies, basing calculations conveniently on a $T\frac{1}{2}$ (half-life) for DDS of one day.[7] Figure 2 is a photograph of the ELISA on these blood spots. Table 2 presents the multiscanner readings. The positive control series indicates that test samples collected up to 4 days after a last daily dose of 100 mg DDS may result in scanner readings lower than 2. Readings of 2–6 are to be expected if the last dose was taken 5–6 days prior to the sampling, and readings of 7–9 indicate that the last dose was taken longer than 6 days ago.

Apart from this ELISA on blood spots which was done in Amsterdam, also

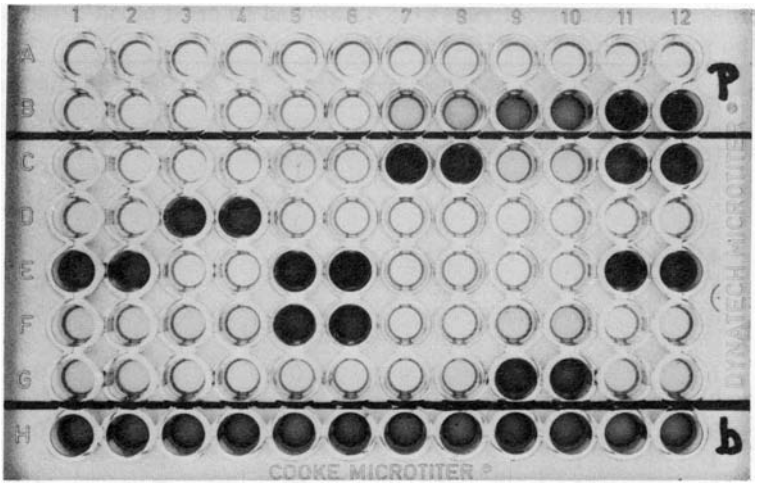

Figure 2. ELISA on blood spots from 30 Ethiopian leprosy patients and controls. All samples are in duplicate. A, 1–12, are 6 positive control samples; B, 1–12, are twofold serial dilutions (in horse serum with 0.05% Tween 20 addition) of pooled positive controls, from 1:2 (1–2) to 1:64 (11–12); C–G, 1–12, are 30 test samples; H, 1–12, are 6 blank control samples.

Table 2. Multiscanner readings of ELISA on blood spots from 30 Ethiopian leprosy patients and controls

	1–2	3–4	5–6	7–8	9–10	11–12	types of samples
A	0	0	0	0	0	0	positive controls
B	0	0	0	2	6	9	positive serial dilutions
C	0	0	0	9	1	$8\frac{1}{2}$	
D	$\frac{1}{2}$	8	0	0	0	$\frac{1}{2}$	
E	$7\frac{1}{2}$	0	9	0	0	9	test samples
F	$\frac{1}{2}$	1	9	0	1	0	
G	1	1	1	1	$8\frac{1}{2}$	1	
H	8	$7\frac{1}{2}$	$7\frac{1}{2}$	$7\frac{1}{2}$	$8\frac{1}{2}$	7	blank controls

The composition of the table corresponds to Figure 2. Numbers are averages of duplicate results. The scanner was set on the optical densities resulting from the blank controls, thus printing these with the matrix numbers 7–9.

urine samples were tested by ELISA in Addis Ababa. Readings of the latter were done by naked eye. Only 4 of the 30 urine samples were negative. In a previous paper[2] it was shown that urine is positive by ELISA up to 4–10 days after a single dose of 100 mg DDS. This means that positive results may be expected up to 5–11 days after a last daily dose of 100 mg DDS.

The blood and urine ELISA results are analysed in Table 3 in relation to the statements of the patients about the dates of their last DDS intake. Table 3 indicates the following:

1. In 19 patients the ELISA tests and the statements correspond.

Table 3. ELISA analysis of DDS self-administration by 30 Ethiopian leprosy patients

Patients numbers	Blood ELISA*	Urine ELISA†	Intake statements‡
19	+	+	+
3	+	+	—
4	—	+	+
4	—	—	+

*Scanner readings ranging from 0–1 were marked +; readings ranging from 7–9 were marked —; other readings did not occur.

†The ELISA on urine was read by naked eye.

‡Statements that the last dose was taken from 0 to 4 days before the sampling are marked +; statements that the last dose was taken more than 6 days before the sampling are marked —; there were no statements that doses were taken on the 5th or 6th day.

2. In 3 patients the ELISA tests disproved the negative statements.
3. In 4 patients the ELISA on blood spots disproved the positive statements, whereas the ELISA on the urine indicated that the last dose could not have been taken longer ago than about 11 days.
4. In 4 other patients both ELISA's disproved the positive statements, thus indicating that the last dose was probably taken longer ago than 11 days.

Discussion

Both ELISA's, but especially the one based on E-DDS, appeared to be sensitive enough for work with dried blood spots. In the E-DDS based ELISA an inhibition of 50% was realized using 25 μl filter paper extract of blood obtained 5–6 days after a single dose of 100 mg DDS. It is remarkable that in both ELISA's finger-prick blood is found to contain a little less sulphones than blood obtained by venapuncture. Since the former type of blood is easily mixed with other tissue fluids, this could mean a confirmation of the recent finding that such fluids contain lower DDS levels than blood.[8] Nevertheless, sulphone levels in finger-prick blood appear to be high enough for detection by ELISA.

As an example of the possible application of this method the examination of 30 blood spots obtained from Ethiopian leprosy patients was described. A choice was made for a semi-quantitative set-up, relating the results to those obtained on serial dilutions of positive control samples. This surely gives more information than the simpler alternative of comparing test samples to undiluted controls only. Those leprologists who are more interested in monitoring the omission of only 1 or 2 daily DDS doses, should make various dilutions of the test samples in order to find out at which dilution a positive sample turns

negative. Conclusions should 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.

A finger prick is an unpleasant experience. Naturally, also blood obtained by an ear-lobe prick can be used in ELISA. An intrinsic advantage of blood over urine is the avoidance of diuresis fluctuations. An advantage of the filter paper technique is its simple management. Certainly it makes the delivery of samples to a central laboratory much easier.

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References

[1] Huikeshoven H, De Wit M, Soeters A, Eggelte TA, Landheer JE, Leiker DL. ELISA inhibition technique for the demonstration of sulphones in body fluids. I. Sulphones specific antibody–enzyme conjugate. *Lepr Rev,* 1979, **50**, 275–81.

[2] Huikeshoven H, De Wit M, Soeters A, Landheer JE, Leiker DL. ELISA inhibition technique for the demonstration of sulphones in body fluids. II. A new method to monitor leprosy patient compliance under field conditions. *Lepr Rev,* 1981, **52**, 11–18.

[3] Ellard GA, Gammon PT, Helmy HS, Rees RJW. Urine tests to monitor the self-administration of dapsone by leprosy patients. *Am J Trop Med Hyg,* 1974, **23**, 464–70.

[4] World Health Organization. The enzyme-linked immunosorbent assay (ELISA). *Bull World Health Organ,* 1976, **54**, 129–39.

[5] Todorovic R, Garcia R. Comparison of the dried blood on filter paper and serum techniques for the diagnosis of bovine babesiosis utilizing the indirect fluorescent antibody (IFA) test. *Tropenmed Parasit,* 1978, **29**, 88–94.

[6] De Wit M, Huikeshoven H, Soeters A, Eggelte TA, Landheer JE, Leiker DL. ELISA inhibition technique for the demonstration of sulphones in body fluids. Comparison of two ELISA methods. *Lepr Rev,* 1981, **52**, 125–130.

[7] Colston MJ, Ellard GA, Gammon PT. Drugs for combined therapy. Experimental studies on the antileprosy activity of ethionamide and prothionamide, and a general review. *Lepr Rev,* 1978, **49**, 115–26.

[8] Halmekoski J, Mattila MJ, Mustakallio KK. Metabolism and haemolytic effect of dapsone and its metabolites in man. *Med Biol,* 1978, **56**, 216–21.