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A Rapid Qualitative Spot Test for the Detection of Dapsone in Urine[1,2]

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The extensive use of dapsone (4,4'-diaminodiphenyl sulfone, DDS) in the treatment of leprosy, especially in patients for whom unsupervised drug therapy may be employed ([3]), has led to the need for a simple and accurate method for monitoring drug intake. The availability of such a method would make routine checks on drug intake feasible.

We have examined a number of spot test color reagents for detecting DDS in urine directly, but have found none that is sufficiently selective for DDS in the presence of normal urinary constituents. Separation of DDS from these interfering substances was effected by extraction of the urine with ethylene dichloride ([2]). Evaporation of the organic solvent and solution of the residue in a small amount of ethanol resulted in an increase in the concentration of DDS of approximately 100-fold over that in the urine. The most specific qualitative spot test for detection of DDS in the ethanolic extraction was found to be the violet color produced by Bratton-Marshall reagents ([5]). The selectivity and accuracy of the spot test technic were tested by concurrent analysis, using thin-layer chromatography (TLC) and spectrophotometry.

MATERIALS AND METHODS

Urine from mature male or female leprosy patients receiving DDS was collected at the various times indicated in the tables. Control urines were collected from mature male and female healthy volunteers without restriction of diet; in most cases, these subjects had not taken any drugs for 24 hours prior to the urine collection. Urine samples from volunteers taking aspirin or other common drugs were collected three hours following drug ingestion. All urine samples were stored in the frozen state before analysis.

Extraction of DDS from urine. A 12 ml. aliquot of urine was treated in a 50 ml. extraction tube with 10 gm. of ammonium sulfate (pyridine-free, Mallinckrodt) and 1 ml. of 50 per cent sodium hydroxide solution (reagent grade, Baker). The mixture (pH 8 to 9) was extracted with 30 ml. of ethylene dichloride (purified grade, Baker)[4] by shaking it mechanically for 15 minutes. After centrifugation to separate the layers, 25 ml. of the organic solvent extract was recovered.

Spot test procedure. A 12.5 ml. aliquot of the ethylene dichloride extract was evaporated to dryness in a vial under a stream of nitrogen. The residue was dissolved in 50 μl. of absolute ethanol, and 5 μl. was applied to filter paper. (This volume of the ethanol extract corresponds to 0.5 ml. of urine.) After five minutes, the spot was sprayed sequentially with 1.0 N hydrochloric acid in 50 per cent ethanol, 0.1 per cent aqueous sodium nitrite solution, 0.5 per

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4 The ethylene dichloride was further purified by passing it through a column (12 x 3 cm.) of acidic alumina, pH 4.2 (Brockman Activity I, 80-200 mesh, Fisher). Its purity was checked as follows: 12.5 ml. was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 50 μl. of absolute ethanol. An aliquot of 5 μl. of the ethanolic solution spotted on filter paper gave no color before or after spraying with Bratton-Marshall reagents as described in the spot test procedure.

TABLE 1. *Tests for DDS on urine from volunteers receiving no treatments or commonly used drugs.*

Sample No.	Treatment	Test results			
		Spot test	TLC		Spectrophotometry (μgm. "DDS"/ml.)
			UV	Color	
C-1 to C-13	None	−	−	−	< 0.1 to 0.2
C-14	None	−	+	−	0.1
C-15 to C-20	Various[a]	−	−	−	< 0.1

[a] Aspirin, phenylephrine hydrochloride or chlorpheniramine maleate.

cent ammonium sulfamate solution in 50 per cent ethanol, and 0.5 per cent N-1-naphthylethylenediamine dihydrochloride in 95 per cent ethanol. A semiquantitative estimation of the amount of DDS was made by comparison with standards. Each series of experimental samples was accompanied by a standard prepared from control urine containing 50 μgm. DDS and by a control urine sample without added DDS. The color intensity obtained in the spot test procedure with the standard was assigned a rating of 4+ (equivalent to 2 μgm. DDS) and the DDS content of the experimental sample was assigned ratings based on the standard. Approximate linearity between intensity of violet color and amount of DDS applied to the paper was noted in the range of 0.05 and 2.0 μgm. DDS.

Thin-layer chromatography. A 5 μl. aliquot of the ethanolic extracts used for the spot test procedure was applied to 20 x 20 cm. Chromagram sheets (Eastman 6061, silica gel), and the chromatogram was developed by the ascending technic in ethyl acetate for 45 minutes[5] and air-dried. DDS was visualized as a bright blue fluorescent spot (R_f 0.72) under exposure to ultraviolet light (254 mμ) and subsequently as a pink-purple spot after spraying with the Bratton-Marshall reagents used for the spot test procedure. The DDS content was estimated by comparison with standards.

[5] We are indebted to Dr. A. J. Glazko for providing the details of the procedure for separating DDS and its N-acetylated derivatives by TLC.

Spectrophotometry. The remaining 12.5 ml. portion of the original ethylene dichloride extract of urine was re-extracted with 5.0 ml. of 1.0 N hydrochloric acid solution, and duplicate 1.0 ml. aliquots of the acid extract were taken for assay of DDS content. To the 1.0 ml. samples contained in a test tube in a water bath at 30 ± 0.5°C. was added 0.5 ml. of 0.1 per cent aqueous sodium nitrite solution. After 15 minutes, 0.5 ml. of 0.5 per cent aqueous ammonium sulfamate was added; three minutes later, 0.5 ml. of 0.5 per cent aqueous N-1-naphthylethylenediamine dihydrochloride was added. Finally, after an additional 30 minutes at 30°C., the samples were removed from the bath and diluted with 2.5 ml. of ethanol. At room temperature, the absorbance of the samples at 560 mμ was determined on the Gilford spectrophotometer. The lower limit of sensitivity in this procedure was found to be approximately 0.1 μgm. DDS/ml. of urine, the range of linearity was between 0.1 and 10 μgm./ml., and the reproducibility of duplicates was approximately ±2 per cent from their mean.

RESULTS

Table 1 presents the results obtained when the spot test procedure and the supporting tests by TLC and spectrophotometry were applied to control urine. It is apparent that all these 20 samples were negative in the spot test and that only one showed a positive test after exposure of the TLC sheet to ultraviolet light. The last

column also shows that the urine blanks under these circumstances were quite low, corresponding to approximately 0.1 μgm. DDS/ml. of urine.

Results of application of the three test procedures to urine from patients under various conditions of therapy with DDS and its repository derivative, 4,-4′-diacetyl-aminodiphenyl sulfone (DADDS) are shown in the subsequent tables. Table 2 shows the results obtained from urine samples provided by patients receiving 50 mgm. DDS daily. All of these samples, which were collected within six hours of the last dose, were found to be clearly positive for DDS by all procedures. No attempt was made to rate the areas of fluorescence after TLC since the intensity of the color test was used for this assessment. The approximate nature of the spot test is emphasized by a comparison between the ratings in the spot test and the actual levels of DDS found *via* spectrophotometry. In only P-9 was a rating made that corresponded to the lower limit of sensitivity. The reason for this was not clear, since the other tests showed the presence of DDS in this sample in amounts that should have given a much higher rating in the spot test. In general, the data of this table show that in urine from patients receiving 50 mgm. DDS daily, the spot test is clearly positive in the majority of the cases tested.

To define more closely the practical limits of the spot test procedure, we collected urine from patients at various times following single doses of 10 and 50 mgm. DDS. The results are given in Table 3. In the two samples obtained at six and 24 hours after the lower dose, positive results were obtained in all tests; at 72 hours however, the results were doubtful. The urine samples obtained after 50 mgm. were, in general, positive for DDS in all tests up to 120 hours (five days) after dosing. Urines collected six or seven days after administration of this dose yielded results in all tests that were close to the limit of sensitivity. Samples P-33, P-40, P-34, P-43, and P-35 were collected at 72, 96, 120, 144 and 168 hours, respectively, from the same patient. It is apparent that the DDS contents estimated semiquantitatively by the spot test were decreasing with increasing time after administration. Similarly, samples P-36, P-42, P-37, P-41 and P-38 were all obtained from another patient at these same intervals after administration. This series showed the same inverse relationship between the spot test ratings and time after administration.

TABLE 2. *Tests for DDS on urine*[a] *from patients receiving 50 mgm. DDS daily.*

Sample No.	Test results			
	Spot test	TLC		Spectrophotometry (μgm. DDS/ml.)
		UV	Color	
P- 1	4+	+	4+	2.3
P- 2	5+	+	5+	2.3
P- 3	6+	+	5+	2.4
P- 4	6+	+	5+	2.0
P- 5	4+	+	5+	2.0
P- 6	5+	+	5+	3.6
P- 7	5+	+	4+	5.7
P- 8	5+	+	6+	13.9
P- 9	+	+	5+	5.2
P-10	5+	+	5+	7.6
P-11	4+	+	5+	8.3
P-15	3+	+	4+	2.8
P-20	6+	+	10+	8.2
P-21	3+	+	4+	2.8
P-22	6+	+	10+	7.9

[a] Urine collected within 6 hours of oral administration.

Although the spot test method was not designed for monitoring patients receiving DADDS, it was of interest to test, for DDS content, the urines from patients receiving DADDS. The data of Table 4 show that four of six samples were positive for DDS by the spot test and that five of these six were positive after TLC by the color test. Nevertheless, all samples contained only minimal quantities of DDS. DADDS was not detected qualitatively by TLC in these urine samples.

DISCUSSION

Although DDS can be extracted into ethylene dichloride from urine in the pH

TABLE 3. *Tests for DDS on urine from patients receiving single doses of 10 or 50 mgm. DDS.*

Sample No.	Dose (mgm.)	Hours after oral administration	Test results			
			Spot test	TLC		Spectrophotometry (μgm. DDS/ml.)
				UV	Color	
P–30	10	6	2+	+	+	0.3
P–12	10	24	2+	+	+	0.4
P–13	10	72	±	−	−	0.2
P–14	50	24	5+	+	4+	1.0
P–19	50	30	+	+	+	0.7
P–23	50	30	2+	+	3+	1.7
P–33	50	72	4+	+	4+	2.0
P–36	50	72	4+	+	2+	1.5
P–39	50	72	+	+	+	0.3
P–40	50	96	2+	+	2+	1.3
P–42	50	96	2+	+	2+	1.0
P–34	50	120	3+	+	2+	0.6
P–37	50	120	3+	+	2+	0.7
P–43	50	144	+	+	+	0.2
P–41	50	144	+	+	+	0.6
P–35	50	168	+	±	+	0.1
P–38	50	168	+	±	+	0.1

range 6.5 to 11, we maintained the pH between 8 and 9 since it was found that under these circumstances blank values for control urine were minimal (Table 1). The efficiency of extraction of DDS from urine by the procedure described was 97 per cent at concentrations ranging from 2 to 10 μgm./ml. and 82 per cent at less than 2 μgm./ml. The purity of the ethylene dichloride was essential since the impurities, if not removed, interfered with the reaction of DDS with the Bratton-Marshall reagents. Other reagents for detecting DDS ([4]), such as 4-dimethylaminobenzaldehyde

TABLE 4. *Tests for DDS on urine from patients receiving 225 mgm. DADDS intramuscularly.*

Sample No.	Days after administration	Test results			
		Spot test	TLC		Spectrophotometry (μgm. DDS/ml.)
			UV	Color	
P–25	4	+	±	+	0.4
P–28	5	−	±	+	0.2
P–17	10	−	−	±	0.1
P–24	25	+	±	+	0.3
P–27	32	+	±	+	0.4
P–31	39	+	±	+	0.1

or 4-dimethylaminocinnamaldehyde, were about equally sensitive to small amounts of DDS, but gave slightly higher blanks, when applied to control urines, than those found by the Bratton-Marshall technic.

The DDS derivatives, monoacetyldiaminodiphenyl sulfone (MADDS) and DADDS, are also extracted from urine under the conditions described, but the spot test procedure was positive only with DDS and MADDS. These two compounds were separated by TLC since MADDS exhibits a lower R_f of 0.56. DADDS, lacking an unsubstituted aromatic amino group, does not give a color with the Bratton-Marshall reagents and therefore does not contribute to the DDS estimations. However, it can be detected by its fluorescence after exposure to ultraviolet light after TLC at an R_f of 0.30. Therefore, only MADDS would be expected to contribute in the spot test for DDS. However, the intensity of color from MADDS, using the Bratton-Marshall reagents, was approximately one-half that obtained with DDS.

The current studies show that patients receiving 50 mgm. DDS daily could be easily monitored for drug intake by the spot test procedure. At a dose of 10 mgm. DDS, only a few urine samples were examined; they were positive in the spot test procedure if collected within 24 hours of drug intake. More detailed studies in patients receiving 50 mgm. DDS indicate that positive results may be expected up to six or seven days after administration; but individual variation may be an important factor since, in one subject, urine obtained three days after drug intake (P-39, Table 3) gave a test result at the limit of sensitivity. Barely detectable amounts of DDS were found in the urine of patients four to 39 days after injection of DADDS.

In general, the results of the qualitative spot test technic were confirmed by TLC and by the more accurate spectrophotometric procedure. Only trace amounts of MADDS were detected by TLC in some of the samples derived from urines of patients receiving DDS. This observation, in addition to the similarities of ratings for DDS in the spot test and in the color test after TLC, suggests that the major compound being measured in the spot test was DDS.

After the current work was completed, a report appeared in which another spot test technic for the qualitative detection of DDS in urine was described ([1]). This method employed tests of urine directly on paper impregnated with a modified Ehrlich reagent (4-dimethylaminobenzaldehyde). Compared with our current procedure, it is less selective, since urea interfered at low DDS concentrations, and less sensitive, since the lower detection limit was approximately 5 μgm. DDS/ml. of urine. However, the author studied a larger number of urine samples collected under more varied DDS-dosage conditions. The relative advantages and disadvantages of the two procedures can no doubt be most rigorously assessed by applying both methods on the same urine samples.

SUMMARY

A rapid spot test procedure for the qualitative detection of DDS in urine of patients receiving this drug is described. To attain maximum selectivity and sensitivity, we extracted the DDS from urine with ethylene dichloride. After evaporation of the solvent, the residue was dissolved in a small amount of ethanol. Aliquots of the ethanolic solution were used for the spot test technic, which was based on the violet color produced by Bratton-Marshall reagents. Supplementary tests by thin-layer chromatography and spectrophotometry confirmed the results of the spot test method. Urine from subjects receiving common remedies or no drugs at all did not contain materials that interfere or give positive results in the spot test technic. Urines obtained up to 24 hours after the ingestion of 10 mgm. DDS or up to 144 to 168 hours after the intake of 50 mgm. DDS were routinely positive. Urines obtained up to 39 days after the intramuscular injection of 225 mgm. DADDS gave test results at the limit of sensitivity of the method, which was approximately 0.1 μgm. DDS per ml. of urine.

RESUMEN

Se describe un procedimiento de "spot test" para la detección cualitativa de DDS en la orina de los pacientes que reciben esta droga. Para conseguir el máximum de selectividad y sensibilidad estrajimos el DDS de la orina, con dicloro ethyleno. Después de la evaporación del solvente, el residuo fué disuelto en una pequeña cantidad de ethanol. Aliquotas de la solución ethanólica fueron usadas para la técnica de "spot test," que fué basada en el color violeta producido por los reactivos de Bratton-Marshall. Exámenes suplementarios por cromatografía en capas delgadas y espectrofotometría confirmaron los resultados del método de "spot test." Orinas de enfermos que recibian remedios comunes o ninguna droga no contenían materiales que interfirieran o dieran resultados positivos en el examen de "spot test." Orinas obtenidas hasta 24 horas después de la ingestión de 10 mgm. de DDS o hasta 144 a 168 horas después de tomar 50 mgm. de DDS fueron rutinariamente positivos. Orinas obtenidos hasta 39 dias después de la inyección intramuscular de 225 mgm. de DADDS dieron resultados que estaban en el límite de sensibilidad del método, el cual fué approximadamente 0.1 μgm. DDS por ml. de orina.

RÉSUMÉ

On décrit ici une épreuve rapide (spot test) pour la détection qualitative de la DDS dans l'urine de malades recevant ce produit. Afin d'atteindre le maximum de sélectivité et de sensibilité, on a extrait la DDS de l'urine par le dichlorure d'éthylène. Après évaporation du solvant, le résidu à été dissout dans une petite quantité d'éthanol. Des aliquots de la solution éthanolique ont été utilisés dans la technique de cette épreuve, qui est basée sur la couleur violette produite par les réactifs de Bratton-Marshall. Des épreuves supplémentaires par chromatographie en couches minces et par la spectrophotométrie ont confirmé les résultats de cette méthode. L'urine de sujets recevant des médicaments usuels, ou bien ne recevant aucun médicament, ne contenait aucune substance susceptible d'interférer ou de fournir des résultats positifs dans épreuve avec cette technique. Les urines obtenues jusqu'à 24 heures après l'ingestion de 10 mg. de DDS, ou jusqua'à 144 à 168 heures après la prise de 50 mg. de DDS, étaient régulièrement positives. Les urines obtenues jusqu'à 39 jours après l'injection intramusculaire de 225 mg. de DADDS fournissaient, à cette épreuve, des résultats qui étaient à la limite de la sensibilité de la méthode, soit approximativement 0.1 μg. de DDS par ml. d'urine.

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