A Fluorometric Method for the Simultaneous Determination of 4,4′-diaminodiphenyl sulfone (DDS), N-acetyl-DDS (MADDS) and N,N′-diacetyl-DDS (DADDS) in Serum or Urine[1,2]

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The demonstration that the growth of *M. leprae* is inhibited by extremely low concentrations of 4,4′-diaminodiphenyl sulfone (DDS) ([15, 16, 18, 22]) and the possibility of treating leprosy with depot injections of DADDS given once every two to three months ([19]), has emphasized the importance of devising sensitive and specific methods for the determination of DDS, N-acetyl-DDS (MADDS) and N, N′-diacetyl-DDS (DADDS). In 1962, Udenfriend ([21]) suggested the possibility of determining DDS fluorometrically by utilizing its native fluorescence. Preliminary studies demonstrated the native fluorescence of DDS, MADDS and DADDS and revealed considerable differences in their fluorescent emission spectra in ethyl acetate. There are also considerable differences in the extent to which these compounds partition between ethyl acetate and dilute hydrochloric acid. Utilizing these properties, a method was devised for the simultaneous determination of all three compounds in serum or urine ([9]). Recently, Glazko and co-workers ([11]) have independently described an extremely sensitive fluorometric method for the determination of DDS and its acid-hydrolizable derivatives. The method described in this paper has been modified from that originally devised ([9]) by using sodium citrate and anhydrous sodium sulfate as recommended by Glazko and his co-workers ([11]).

METHODS

Analytical grade reagents were dissolved in glass distilled, de-ionized water. Analytic grade ethyl acetate was distilled to remove nonvolatile fluorescent impurities. Aqueous solutions of M tri-sodium citrate, 0.1 N sodium hydroxide and hydrochloric acid were purified by extracting with 2 x 0.1 volume ethyl acetate. For the most accurate studies, all glassware was first washed with ethyl acetate ([11]).

The multiple extraction procedure used for the fluorometric determination of DDS, MADDS and DADDS is illustrated diagrammatically in Figure 1. Three ml. of serum or urine was extracted by shaking for 10 seconds with 15 ml. ethyl acetate and 1 ml. M tri-sodium citrate in a stoppered centrifuge tube on a Vortex mixer. The phases were separated by centrifugation and the ethyl acetate extract removed using a Pasteur pipette. Twelve ml. of the ethyl acetate extract was then washed by shaking with 1 ml. 0.1 N sodium hydroxide and 10 ml. of this washed extract was further washed by shaking with 1 ml. 0.1 N hydrochloric acid.

Eight ml. of the washed ethyl acetate extract was then extracted by shaking with 2 ml. 1.2 N hydrochloric acid. Under these conditions, the great majority of the DDS was extracted into the 1.2 N hydrochloric acid, while MADDS and DADDS remained in the ethyl acetate phase. One ml. of the 1.2 N hydrochloric acid extract was shaken with 2 ml. M sodium citrate and 2 ml. ethyl acetate, and the ethyl acetate extract dried by shaking with 0.5 gm. anhydrous sodium sulphate .DDS was then determined by measuring the fluorescence of the extract at 345 mμ in an Aminco-

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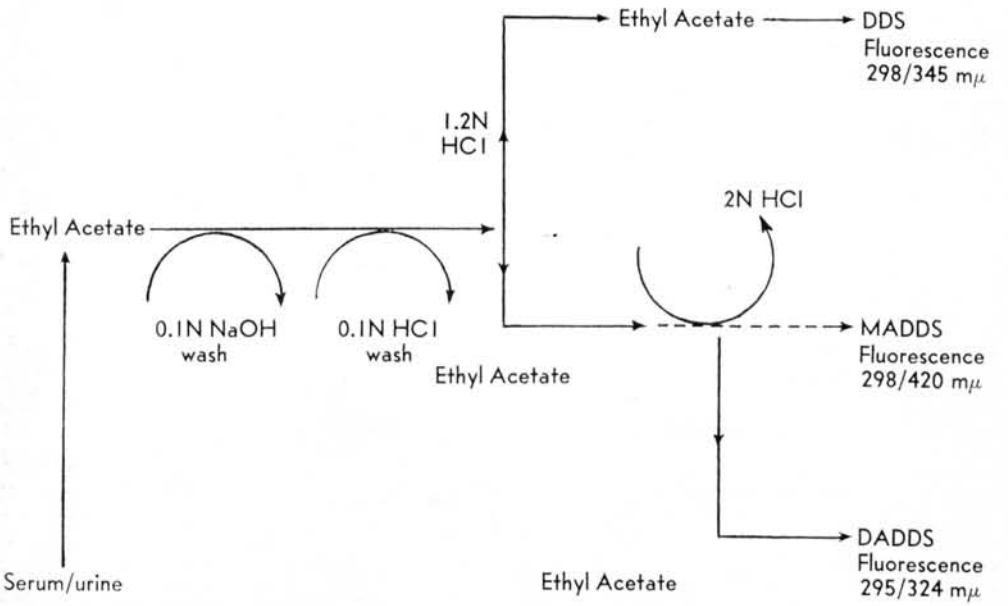

Fig. 1. Extraction and fluorometric determination of DDS, MADDS and DADDS.

Bowman spectrophotofluorometer, the excitation wavelength being 298 mμ.

MADDS was determined by shaking 6 ml. of the original ethyl acetate extract with 1 ml. M sodium citrate, drying with 0.5 gm. anhydrous sodium sulfate and measuring the fluorescence at 420 mμ, the excitation wavelength being 298 mμ.

Four ml. of this dried ethyl acetate extract was then shaken with 5 ml. 2 N hydrochloric acid to remove the majority of the MADDS. Two ml. of this washed ethyl acetate extract was shaken with 1 ml. M sodium citrate, dried with 0.5 gm. anhydrous sodium sulfate, and DADDS determined by measuring the fluorescence at 324 mμ, the excitation wavelength being 295 mμ.

Significant concentration-quenching occurred when more than about 10 μg of DDS, MADDS or DADDS were initially present, and in this case, the final extracts were diluted with the appropriate blank extracts so that the final concentration did not exceed 1 μg/ml. before measuring the fluorescence.

This method was applied to a preliminary study of the serum concentrations and urinary excretion of DDS, MADDS and DADDS in six Caucasian patients suffering from dermatitis herpetiformis, who were being treated for long periods with daily doses of from 50 to 300 mgm.DDS. It was also used to measure the urinary excretion of DDS, its acid-labile conjugates, and MADDS and DADDS by a healthy Caucasian subject after a single oral dose of 100 mgm. DDS, given alone or with concomitant potassium citrate (4 x 3 gm. daily) to render the urine neutral. This subject was a slow acetylator of sulfamethazine and isoniazid. Before assay, all urine samples were diluted to a volume equivalent to a urinary excretion of 100 ml./hr. DDS plus its acid-labile conjugates were determined as DDS after adding 0.5 ml. N hydrochloric acid to 2 ml. urine, allowing it to stand for one hour at room temperature (23°C) and neutralizing by the addition of 0.5 ml. N. sodium hydroxide.

RESULTS

The recovery of each compound from either serum or urine was identical to that from aqueous solution. It was not possible to calculate the absolute recovery of each compound because of volume changes in the phases during the multiple extraction steps. However, the final concentration of DDS in the ethyl acetate extract used to determine its fluorescence was about 32 per cent of its initial concentration in serum or

urine; of MADDS, about 17 per cent of its initial concentration, and of DADDS, about 23 per cent of its initial concentration. Serum blanks were negligible and the method could be used to measure concentrations of down to about 0.02 μgm./ml DDS, 0.1 μgm./ml. MADDS and 0.02 μgm./ml. DADDS, respectively, in serum.[4] When applied to urine, the sensitivity of the method was largely determined by the size and constancy of the blank values, which were normally equivalent to an excretion of about 0.2 mgm./day of each compound.

The specificity of the method for the determination of DDS and DADDS was of a high order, but both DDS and DADDS interfered to an appreciable extent in the determination of MADDS (Table 1). Further evidence as to the identity of the compounds that this method was actually measuring could be obtained by measuring the fluorescence of the extracts at several wave-lengths and comparing the results with those from the standards. In this study, the wave-lengths employed were those at which the DDS, MADDS and DADDS extracts, respectively, fluoresced maximally. The ratios of fluorescence at these wave-lengths for each compound are shown in Table 2.

In patients on continuous therapy, the concentrations of DDS found in the serum immediately before daily dosage with the drug averaged 1.2 μgm./ml./100 mgm/DDS/day. Although there was very little day to day variation in DDS serum concentrations, the urinary excretion of DDS varied considerably from day to day, and averaged 16.6 ± 8.8 per cent of the dose. Confirmation of the identity of the compound extracted from the serum and urine samples by the DDS method was obtained by demonstrating that its fluorescence was unaffected by heating the 1.2 N hydrochloric acid extract for one hour at 100°C, but was destroyed by treating with 1 per cent sodium nitrite (0.1 vol.) for five minutes at room temperature. This latter technic has been used to provide individual serum and urine blanks in an investigation of the serum concentrations and urinary excretion of DDS by leprosy patients being treated with only one mgm. DDS a day ([22]).

In the study of the effect of urinary pH on the excretion of DDS, when the pH of the urine was normal (6.1 ± 0.3), 11 per cent of the dose was excreted unchanged and 14 per cent as acid-labile conjugates of DDS within four days. When potassium citrate was given concomitantly so that the pH of the 24 hour urine collections was 7.3 ± 0.2, only 5 per cent of the dose was excreted unchanged, and 17 per cent was excreted as acid-labile conjugates of DDS. The results are summarized in Table 4. The kinetics of the fall with time in the urinary excretion of DDS and its acid-labile con-

TABLE 1. *Specificity of the fluorometric method for the determination of DDS, MADDS and DADDS.*

Method for determination of	Relative fluorescence of compounds		
	DDS	MADDS	DADDS
DDS	100	4	1
MADDS	14	100	14
DADDS	<0.1	<0.2	100

TABLE 2. *Emission fluorescence characteristics of DADDS, MADDS and DADDS.*

Compound	Ratio[a] 420/345 mμ	Ratio[b] 324/345 mμ
DDS	0.27	0.39
MADDS	22.0	0.2
DADDS	0.04	1.4

[a] Ratio $\frac{\text{fluorescence at wave-lengths maximal for MADDS}}{\text{fluorescence at wave-lengths maximal for DDS}}$

[b] Ratio $\frac{\text{fluorescence at wave-lengths maximal for DADDS}}{\text{fluorescence at wave-lengths maximal for DDS}}$

[4] Lower limits of sensitivity were based on the variations of reagent blanks and defined as the amounts of compounds giving net readings equal to twice the standard deviations of their respective blanks.

jugates were similar whether or not citrate was given and were equivalent to a half-life of about 18-19 hours. The fluorescence characteristics of the compound liberated by acid-hydrolysis at room temperature were identical to those of DDS (Table 3).

The concentrations of MADDS and DADDS found in the serum of patients on continuous therapy immediately before daily dosage with DDS, averaged 0.3 μgm./ml. and 0.2 μgm./ml., respectively, per 100 mgm. daily DDS. The compound estimated as MADDS was, like MADDS, removed by washing with 2N hydrochloric acid, and its fluorescence was destroyed by nitrous acid. It could also be converted by acid-hydrolysis to a compound that extracted and fluoresced like DDS. The compound estimated as DADDS was, like DADDS, unaffected by treatment with nitrous acid and could be converted by acid-hydrolysis to a compound that extracted and fluoresced like DDS. Although it was probable that both MADDS and DADDS were present in the serum after dosage with DDS, the fluorescent characteristics of these MADDS- and DADDS-like compounds could not be established separately

TABLE 3. *Fluorescence characteristics of the compound extracted from serum and urine into 1.2N hydrochloric acid after dosage with DDS.*

Sample	Ratio fluorescence 420/345 mμ	Ratio fluorescence 324/345 mμ
During continuous DDS dosage		
Serum extracts (25)[a]	0.28 ± 0.03[b]	0.41 ± 0.02
DDS standards	0.27 ± 0.02	0.39 ± 0.01
Urine extracts (21)[a]	0.26 ± 0.02	0.41 ± 0.01
DDS standards	0.25 ± 0.02	0.39 ± 0.01
After single dose 100 mgm. DDS		
Urine extracts (15)[a]	0.28 ± 0.01	0.39 ± 0.02
DDS standards	0.28 ± 0.01	0.39 ± 0.01
Acid-hydrolyzed		
urine extracts (12)[a]	0.29 ± 0.02	0.40 ± 0.01
DDS standards	0.28 ± 0.01	0.40 ± 0.01

[a] Number of samples
[b] Mean and standard deviation of observations.

TABLE 4. *Urinary excretion of DDS and its acid-labile conjugates (mgm.) after dosage with 100 mgm. DDS.*

	DDS given alone			DDS given with citrate		
	DDS	DDS plus conjugates	Difference (conjugates)	DDS	DDS plus conjugates	Difference (conjugates)
Day 1	6.55	12.9	6.3	2.75	12.5	9.7
Day 2	2.77	6.8	4.0	1.40	6.5	5.1
Day 3	1.25	3.4	2.2	0.44	1.8	1.4
Day 4	0.43	1.5	1.1	0.21	1.0	0.8
TOTAL	11.00	24.6	13.6	4.80	21.8	17.0

by this method, so that their fluorescence could not be directly compared with that of authentic MADDS or DADDS.

Neither MADDS nor DADDS could be detected in the urine after single 100 mgm. doses of DDS given with or without citrate. When the method was applied to analyze the urine samples collected from the dermatitis herpetiformis patients, the results were less conclusive, since pretreatment samples of blank urine could not be obtained. However, these results indicated that the urinary excretion of MADDS and DADDS could not have exceeded 1 per cent of the dose.

DISCUSSION

This method, which is capable of determining concentrations of down to about 0.02 μgm./ml. DDS in serum and an excretion of 0.2 mgm. DDS per day in urine, is considerably more sensitive and specific than the colorimetric methods at present available for the determination of DDS. By measuring the fluorescence of the DDS extracts at several wave-lengths, it was shown that the fluorescence characteristics of the extracts of serum and urine from patients being treated with DDS were identical to those of the unchanged drug. It was noted that serum samples could be stored for periods of up to six months at —20°C, without any significant change in their DDS content.

The concentrations of DDS found in the serum of patients on continuous therapy immediately before daily dosage with the drug, averaged about 1.2 μgm./100 mgm. daily DDS. This concentration is almost identical to that expected from the results obtained by Glazko and his co-workers [11] with their fundamentally similar fluorometric method, when peak plasma concentrations of about 1.2 μgm./ml. were found after a single 100 mgm. dose of DDS, and then fell, with an average half-life of 20.6 hours. Since the concentrations of MADDS and DADDS found in the serum of these patients immediately before daily dosage with DDS averaged 0.3 μgm./ml. and 0.2 μgm./ml. respectively, per 100 mgm. DDS, the ratio of free plus acetylated drug to free DDS averaged 1.4. This may be compared with the results of Chang [7] and Glazko [10] and their co-workers, who, using their fluorometric method [11], found ratios of acid-hydrolizable to free DDS of 1.7—1.8 in the plasma of subjects who had been given a single intramuscular dose of DADDS. In the rhesus monkey, they found ratios of about 20 after oral doses of DDS, or intramuscular doses of DADDS, indicating concurrent processes of acetylation and hydrolysis.

The urinary excretion of unchanged DDS by the patients averaged 17 per cent of the dose, which is similar to that determined previously when colorimetric methods were employed [8, 13, 17, 19]. Although there was little variation in DDS serum concentrations from day to day, there was considerable daily variation in the urinary excretion of unchanged DDS. A possible explanation for this variation that has also been encountered by Shepard and his co-workers [19], is the hypothesis of Bushby [3] that the amount of DDS excreted unchanged may be directly related to the extent to which a major metabolite of the drug, its acid-labile N glucuronide [5, 6, 12, 20], is spontaneously broken down in the urine. Bushby and Woiwod [5], have shown that when potassium citrate was given concomitantly in a total daily dose of 35 gm. [4] to raise the pH of the urine to about eight [3], less than 5 per cent of the dose was excreted unchanged.

This hypothesis was tested by examining the effect of giving 4 x 3 gm. potassium citrate daily on the excretion of DDS and its acid-labile conjugates. Giving citrate increased the average pH of the urine from 6.1 to 7.3. It reduced the excretion of unchanged DDS from 11 per cent to 5 per cent of the dose, but this was accompanied by a slightly increased excretion of acid-labile conjugates of DDS. These findings support Bushby's hypothesis and argue against a previous suggestion by one of us (G.A.E.) that the N-glucuronide of DDS might be spontaneously formed in the urine [8], as occurs with some other aromatic amines [2]. The possibility of increased reabsorption of DDS by the kidney at higher urinary pHs cannot, however, be

entirely discounted, as thi sis known to occur with basic drugs such as amphetamine (1).

The half-life of DDS in the serum was determined in one of the patients (17 hours) and was similar to the half-life of the urinary excretion of both DDS and its acid-labile conjugates (18-19 hours) in the healthy subject, and to the values previously obtained by Glazko and his co-workers (11).

Although there was evidence for the presence of MADDS and DADDS in serum after dosage with DDS, these compounds could not be detected in the urine. Earlier colorimetric studies (8) showed that about 27 per cent of the dose of DDS was excreted in the urine as compounds that were resistant to acid hydrolysis at room temperature but could be converted to a diazotizable amine that extracted and reacted like DDS after boiling for one hour with N hydrochloric acid, and it was postulated that these metabolites might be acetylated derivatives of DDS or acid-labile glucuronides of the drug. These compounds could not be extracted from the urine with methyl isobutyl ketone (unpublished results). Since that work was carried out, synthetic samples of MADDS and DADDS have become available, and it has been found that they would have been completely extracted with this solvent. Hence, it must be concluded that these compounds could not have been MADDS or DADDS. Recently, Peters and his co-workers (14) have described a spot test for the detection of DDS in urine, and have used a thin layer chromatographic procedure originally devised by Glazko to study the urinary excretion of DDS, MADDS and DADDS after dosage with DDS or DADDS. Although DDS was readily detected in the urine after oral dosage with the drug, only traces of MADDS were excreted and there was no report of the excretion of DADDS in the urine.

The apparent anomaly of our detecting MADDS and DADDS in the serum but not in the urine, after dosage with DDS, could be explained if it were assumed that the reabsorption of MADDS and DADDS by the kidney was even greater than that of DDS, which, on the available evidence, is probably at least 98 per cent.

SUMMARY

A fluorometric method has been devised for the simultaneous determination of DDS, MADDS and DADDS in serum or urine, based on differences in their native fluorescence in ethyl acetate and their partition between ethyl acetate and dilute hydrochloric acid. The method is capable of measuring concentrations of down to about 0.02 μgm./ml. DDS, 0.1 μgm./ml. MADDS and 0.02 μgm./ml. DADDS, respectively, in serum, and a daily urinary excretion of 0.2 mgm. of each compound.

The method has been used to study the acetylation of DDS by a small group of dermatitis herpetiformis patients on continuous DDS therapy. Immediately before daily dosage with DDS, serum concentrations averaged 1.2 μgm./ml. DDS, 0.3 μgm./ml. MADDS and 0.2 μgm./ml. DADDS, respectively, per 100 mgm. daily DDS dose. Urinary excretion of unchanged DDS averaged 17 per cent of the dose, but neither MADDS nor DADDS could be detected in the urine.

The effect of the pH of the urine on the excretion of DDS and its acid-labile conjugates was also studied. Raising the pH of the urine from 6 to 7 did not alter the total excretion of DDS plus its acid-labile conjugates, but led to a significant reduction in the excretion of unchanged DDS.

RESUMEN

Se ha desarrollado un método fluorométrico para la determinación simultánea de DDS, MADDS y DADDS en suero y orina, basado en diferencias de su fluorescencia natuarl en acetato de etilo y su separación entre acetato de etilo y ácido chorhídrico diluído. El método es capaz de medir concentraciones hasta de alrededor de 0.02 μg/ml. DDS, 0.1 μg/m. MADDS y 0.02 μg/ml. DADDS en el suero y una excreción diaria urinaria de 0.2 mgm. de cada compuesto. El método ha sido usado para estuliar la acetilación del DDS en un pe-

queño grupo de enfermos de dermatitis herpetiformis en tratamiento contínuo con DDS. Inmediatamente antes de la dosis diaria de DDS, la concentración media sérica fué de 1.2 μg/ml. DDS, 0.3 μg/ml. MADDS y 0.2 μg/ml DADDS respectivamente, para 100 mgm. diarios de DDS. La excreción urinaria de DDS no modificado tuvo una media de 17 por ciento de la dosis pero ni MADDS ni DADDS pudieron ser detectados en la orina.

Se estudió también el efecto del pH urinario sobre la excreción de DDS y sus conjugados ácido-lábiles. El aumento del pH de la orina de 6 a 7 no alteró la excreción total de DDS más sus conjugados ácido-lábiles, pero produjo una reducción significativa en la excreción del DDS no transformado.

RÉSUMÉ

Invention d'une méthode fluorométrique pour la détermination simultaneé des DDS, MADDS et DADDS dans le sérum ou dans l'urine; cette méthode est fondeé sur les différences de fluorescence native de ces substances dans l'acétate d'éthyle et leur séparation par ultisation d'acétate d'éthyle et d'acide cholorhyrique dilué. Il est ainsi possible de mesurer des concentrations allant jus'qu á 0.02 microgrammes/ml. de DDS, 0.01 microgrammes/ml. de DADDS dans le sérum, et de 0.2 milligrammes de chacune de ces substances dans l'excrétion urinaire d'une journée.

La méthode a aussi été utilisé pour étudier l'acétylation de la DDS chez un petit groupe de malades atteints de dermatite herpétiforme et traités de manière continuelle par ce medicament. Immediatement avant l'administration de la dose quotidienne de 100 milligrammes, les concentrations seriques moyennes étaient les suivantes: DDS, 1.2 microgrammes/ml.; MADDS, 0.3 microgrammes/ml.; DADDS, 0.2 microgrammes/ml.

L'excrétion urinaire de DDS non modifiée atteignait en moyenne 17% de la dose administrée, mais les auteurs r'ontper déceler dans l'urine ni la MADDS ni la DADDS.

L'effet du pH de l'urine sur l'excretion de la DDS et ses conjugés acide labiles a aussi été étudié. L'elevation du pH de l'urine de 6 á 7 ne modifie pars l'excrétion totale de la DDS et de ses conjugés acido labiles, mais entraîne un réduction significative de l'excrétion de la DDS non modifieé.

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