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

G. A. Ellard and Patricia T. Gammon

METHODS

Analytical grade reagents were dissolved in glass distilled, de-ionized water. Analytical grade ethyl acetate was distilled to remove non-volatile 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.

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 stopped 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 nm in an Amino-
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 7 gm. daily) to render the urine neutral. This subject was a slow acetylator of sulfanamethazine and iodonizid. 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 μg/ml DDS, 0.1 μg/ml MADDS and 0.02 μg/ml DADDS, respectively, in serum. 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 excetration of about 0.2 mg/m.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 dosages with the drug averaged 1.2 μg/ml/100 mg 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 ± 5.8 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, being treated with only 0.1 mg sodium nitrite (0.1 vol.) for five minutes at room temperature. This latter technique 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 mg DDS a day (2).

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-

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

<table>
<thead>
<tr>
<th>Method for determination of</th>
<th>DDS</th>
<th>MADDS</th>
<th>DADDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDS</td>
<td>100</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>MADDS</td>
<td>&gt;0.1</td>
<td>&lt;0.2</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2. Emission fluorescence characteristics of DADDS, MADDS and DDS.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio⁺</th>
<th>Ratio⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDS</td>
<td>420/345</td>
<td>0.27</td>
</tr>
<tr>
<td>MADDS</td>
<td>224/345</td>
<td>0.2</td>
</tr>
<tr>
<td>DADDS</td>
<td>0.04</td>
<td>1.4</td>
</tr>
</tbody>
</table>

⁺ Ratio of fluorescence at wave-length maximal for MADDs to fluorescence at wave-length maximal for DDS.
⁻ Ratio of fluorescence at wave-length maximal for DDS to fluorescence at wave-length maximal for MADDs.

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 mgm./ml. and 0.2 mgm./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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio fluorescence</th>
<th>Ratio fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>420/345 mg</td>
<td>324/345 mg</td>
</tr>
<tr>
<td>During continuous DDS dosage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum extracts (25)</td>
<td>0.28 ± 0.03</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>DDS standards</td>
<td>0.57 ± 0.02</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Urine extracts (21)</td>
<td>0.26 ± 0.02</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>DDS standards</td>
<td>0.25 ± 0.02</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>After single dose 100 mgm. DDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine extracts (15)</td>
<td>0.28 ± 0.01</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>DDS standards</td>
<td>0.28 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Acid-hydrolyzed urine extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>0.28 ± 0.02</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>DDS standards</td>
<td>0.28 ± 0.02</td>
<td>0.40 ± 0.01</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>DDS given alone</th>
<th>DDS given with citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDS</td>
<td>DDS plus conjugates</td>
</tr>
<tr>
<td>Day 1</td>
<td>6.55</td>
</tr>
<tr>
<td>Day 2</td>
<td>2.77</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.25</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.43</td>
</tr>
<tr>
<td>TOTAL</td>
<td>11.00</td>
</tr>
</tbody>
</table>
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 mgm./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 mgm./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 fluorimetric method, when peak plasma concentrations of about 1.2 mgm./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 mgm./ml. and 0.2 mgm./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 fluorimetric method (10), found ratios of acid-hydrolyzable 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 (6, 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 (4) 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, 10, 18), is spontaneously broken down in the urine. Bushby and Wooton (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 (8), 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. Bushby 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 (4), as occurs with some other aromatic amines (7). The possibility of increased reabsorption of DDS by the kidney at higher urinary pHs cannot, however, be
entirely discounted, as this is known to occur with basic drugs such as amphetamine (8).

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 Clazko and his co-workers (19).

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 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 (11) have described a spot test for the detection of DDS in urine, and have used a thin layer chromatographic procedure originally devised by Clazko 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 µg/ml DDS, 0.1 µg/ml MADDS and 0.02 µg/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 µg/ml DDS, 0.3 µg/ml MADDS and 0.2 µg/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 nativa en acetato de etilo y su separación entre acetato de etilo y ácido clorhídrico diluido. El método es capaz de medir concentraciones hasta de alrededor de 0.02 µg/ml DDS, 0.1 µg/ml 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 estudiar la acetilación del DDS en un pe-
quien grupo de enfermos de dermatitis herpetiforme en tratamiento continuo con DDS, inmediatamente antes de la dosis diaria de DDS, la concentración media sérica fue de 1.5 μg/ml. DDS, 0.3 μg/ml MADD y 0.2 μg/ml DADD respectivamente, para 100 mg/dos días de DDS. La excreción urinaria de DDS no modificada tuvo una media de 17 mg/día, pero en DDS ni MADD ni DADD pudieron ser detectados en la orina.

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

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**REFERENCES**


Ellard & Gammon: Method for Determination of DDS, MADDS & DADDS


