A FLUOROMETRIC METHOD FOR THE DETERMINATION
OF PAMAQUINE, SN-13276, AND SN-3294*

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Brodie et al. (1) have described a photometric method for the determination of pamaquine which is based upon the coupling of this compound with diazotized sulfanilic acid. This procedure is satisfactory, but alternative methods are desirable, particularly for studies of the disposition of this antimalarial compound in the tissues and body fluids of various animals. Alternative analytical methods, based upon different properties of a therapeutic compound, provide information concerning the degradation and subsequent disposal of the drug which a single method cannot yield so readily. The fluorescence of pamaquine, SN-13276, and SN-3294 in concentrated sulfuric acid provides the basis for the analytical method described in the present paper.

**Analytical Procedure**

**Reagents—**

1. Stock standard solution of pamaquine, 100 mg. per liter. Dissolve 161 mg. of the citrate salt in 0.1 N sulfuric acid, and dilute the solution to 1 liter with the acid. This solution is stable for several weeks when stored in a refrigerator. Working standards are prepared daily by dilution with 0.1 N H₂SO₄.

2. 0.1 N NaOH.

3. 1.0 N NaOH.

4. Hexane. Eastman Kodak Company, practical grade (from petroleum). This crude solvent contains both volatile and non-volatile substances which fluoresce in concentrated sulfuric acid. The solvent is

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1 Pamaquine (plasmochin) is 6-methoxy-8-(4'-diethylamino-1'-methylbutylamino)-quinoline. Numbers preceded by the letters, SN, are the code numbers assigned by the Office of the Survey of Antimalarial Drugs. SN-13276 is 6-methoxy-8-(5'-isopropylaminoamylamino)-quinoline. SN-3294 is 6-methoxy-4-(4'-diethyl-amino-1'-methylbutylamino)-quinoline.

* We are indebted to Dr. Leslie Hellerman and Dr. Curt C. Porter for a sample of purified pamaquine citrate prepared, by their unpublished procedure, from a commercial sample of pamaquine.
freed of the non-volatile substances by distillation in an all-glass apparatus at atmospheric pressure. Collect the fraction boiling from 66–72° and store it in a glass-stoppered bottle. The volatile, fluorescent substances do not interfere in the method described below, since the final hexane extract is evaporated prior to addition of sulfuric acid.

5. n-Butyl alcohol, reagent grade.
6. 0.1 N HCl.
7. Sulfuric acid, c. p., analytical reagent grade, specific gravity 1.84.

Most samples of concentrated sulfuric acid exhibit some fluorescence when irradiated by means of a mercury vapor lamp. However, "blank" fluorescence from this source is quite constant for a particular batch of the acid, and corrections are readily made in the calculations.

Procedure—In the following procedure pamaquine is specified, but the same directions apply in the determination of SN-13276.

Add 1 to 10 ml. of plasma or other biological sample (containing 0.2 to 5 γ of pamaquine) and an equal volume of 0.1 N NaOH to 30 ml. of redistilled hexane in a glass-stoppered bottle. Shake the mixture for 15 minutes on a mechanical shaker. Centrifuge to separate the phases as completely as possible. If a thick layer of emulsion exists between the phases after the first centrifugation, break the emulsion with a stirring rod and repeat the centrifugation. Add 0.5 ml. of n-butyl alcohol to the hexane phase with minimum disturbance of the aqueous layer. With a pipette transfer exactly 25 ml. (or, if necessary, a smaller volume accurately measured) of the hexane phase to a small, tapered separatory funnel; add 5 ml. of 0.1 N HCl and shake vigorously for several minutes. Allow the phases to separate; then transfer the entire acid aqueous phase to another separatory funnel. Extract the hexane again with 2 ml. of 0.1 N HCl and combine this second acid extract with the first. Introduce 25 ml. of redistilled hexane into the funnel containing the acid extracts. Add 1 ml. of 1.0 N NaOH and shake the mixture vigorously for 5 minutes. Allow the phases to separate and add 0.5 ml. of n-butyl alcohol to the hexane with minimum disturbance of the aqueous layer. Draw off the aqueous phase and discard. Transfer the hexane quantitatively to a 100 ml. distilling flask and evaporate to dryness by distillation under diminished pressure (water aspirator pump) in an all-glass still. Maintain the water bath in which the distilling flask is immersed at 30–50° during the distillation. It is important to remove all traces of the solvent from the flask. Then place exactly 8 ml. (or slightly more than the minimum volume required by guest on August 14, 2017 http://www.jbc.org/ Downloaded from
for the particular fluorometer employed) of concentrated sulfuric acid in
the distilling flask and spread the acid over the entire inner surface of the
flask in order to bring all of the pamaquine into solution. Transfer the
acid to a cuvette and determine the fluorescence in a fluorometer. The
irradiation of the sample is best carried out by means of a mercury vapor
lamp. For the greatest sensitivity, Corning Filter 5970 (new code number
of the Corning Glass Works), specially ground to a thickness of 1 mm.,
should be placed between the lamp and the cuvette. However, Coleman
Filter B5S also can be used in this “primary” position with a small de-
crease in analytical sensitivity. The secondary filter inserted between the
cuvette and the photocell should be Corning Filter 3060 or 3389 or Cole-
man Filter PC. Filter 3060 will provide greatest analytical sensitivity,
but with fluorometers in which a considerable amount of the primary ra-
diation reaches the photocell by reflection from the sides of the cuvettes
or by other sources of “scattering,” it may be necessary to use Filter 3389
instead of Filter 3060 to minimize the blank from this source. Also, when
blanks due to scattered radiation are serious, it may be necessary to use a
combination of Filters 3389 and 5030 in the secondary position. Filter
5030 blocks the radiation in the “red” range of the spectrum which also is
transmitted by primary Filter 5970. Transmittance-wave-length curves
for these filters are presented in “Glass color filters,” a publication of the
Corning Glass Works.

Standards should be carried through the entire procedure with each set
of unknowns, and the concentrations of the latter are calculated from the
fluorometric values of these standards. A reagent blank, which includes
the blank due to the sulfuric acid, should be determined by substituting
distilled water for the biological sample in the procedure. In the calcula-
tions, the fluorometric value of the reagent blank is deducted from the values
for standards and unknowns. The intensity of fluorescence of the stand-
ards is directly proportional to the concentration of pamaquine.

The same procedure can be used for the determination of SN-3294 in
amounts as small as 0.1 γ; but analytical recoveries are more nearly quanti-
tative if freshly redistilled ethylene dichloride (1,2-dichloroethane) is
used in place of hexane for the extraction of this compound.

RESULTS AND DISCUSSION

It is shown in Table I that pamaquine and SN-3294 added to plasma
are recoverable with adequate precision by this analytical procedure. The
method is equally applicable to the determination of SN-13276. The
amount of interfering fluorescent material in normal plasma is negligible
when the analysis is carried out by the double extraction procedure de-

6 Coleman photofluorometer, or Klett fluorometer, or other similar instrument.
scribed above. Attempts to shorten the method by extracting pamaquine directly from the initial organic solvent extract with concentrated sulfuric acid, or by evaporating the initial extract and adding sulfuric acid, were unsatisfactory owing to excessive "blank" fluorescence derived both from the solvent and from normal plasma under these conditions. When the double extraction procedure is used as described, the reagent blank is not negligible but is quite constant and reproducible with a particular batch of reagents. In order to minimize the blank, it is particularly important to distil the solvents prior to use.

An attempt was made to determine the basis for the fluorescence of pamaquine and SN-3294 in concentrated sulfuric acid. As presented in

### Table I

**Analytical Recovery of Pamaquine and SN-3294 from Plasma**

Data for recoveries are the averages of five determinations at each concentration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of compound in plasma (theoretical)</th>
<th>Volume of plasma sample analyzed</th>
<th>Average concentration of compound in plasma (found)</th>
<th>Average recovery</th>
<th>Average deviation of single determination from average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ per l.</td>
<td>ml.</td>
<td>γ per l.</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>Pamaquine</td>
<td>20</td>
<td>10</td>
<td>18.4</td>
<td>92.0</td>
<td>±10.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>47.2</td>
<td>94.4</td>
<td>±8.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>95.2</td>
<td>95.2</td>
<td>±6.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5</td>
<td>195.0</td>
<td>97.5</td>
<td>±5.0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2</td>
<td>490.0</td>
<td>98.0</td>
<td>±4.8</td>
</tr>
<tr>
<td>SN-3294</td>
<td>10</td>
<td>10</td>
<td>8.9</td>
<td>89.0</td>
<td>±12.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10</td>
<td>18.7</td>
<td>93.5</td>
<td>±9.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>48.1</td>
<td>96.2</td>
<td>±7.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>95.3</td>
<td>95.3</td>
<td>±5.5</td>
</tr>
</tbody>
</table>

the preceding paper (3), spectrophotometric and potentiometric evidence demonstrated that in 7 M sulfuric acid pamaquine is a tripolar cation with protons attached to the diethylamino nitrogen of the side chain, the quinoline ring nitrogen, and the secondary 8-amino nitrogen. When the concentration of sulfuric acid is increased from 8 to 18 M, there is a small shift in the absorption spectrum of pamaquine (Fig. 1). Solutions of pamaquine in 7 M sulfuric acid are practically non-fluorescent when irradiated by sunlight or by the 365 mµ line of the mercury emission spectrum. As the concentration of sulfuric acid is increased from 8 to 18 M, there is a progressive increase in the fluorescence, solutions of pamaquine in 18 M sulfuric acid exhibiting brilliant violet-blue fluorescence. The mid-point of the change is in 13.76 M sulfuric acid. These changes in spectrophotometric absorption and fluorescence might be attributed to "medium effects," i.e. to
progressive changes in properties of the medium such as density and viscosity, but there would seem to be a possibility that the changes are due, at least in part, to a proton exchange involving the methoxy group of pamaquine as proton acceptor. The fact that the changes in absorption and fluorescence can be reversed by dilution of the concentrated acid solutions with water is in favor of the interpretation of the changes as due to a reversible proton exchange. In favor of the conclusion that the methoxy group is involved is the fact that desmethoxy pamaquine (8-(4'-diethyl-

![Figure 1. Change in spectrophotometric absorption by pamaquine in aqueous sulfuric acid from 6 to 17.4 M.](http://www.jbc.org/) Downloaded from http://www.jbc.org/ by guest on August 14, 2017

amino-1'-methylbutylamino)-quinoline) is practically non-fluorescent in both 8 M and 18 M sulfuric acid.

Inasmuch as quinine also is a derivative of 6-methoxyquinoline, it was desirable to study the spectrophotometric absorption and fluorescence of this compound in concentrated aqueous solutions of sulfuric acid for comparison with pamaquine. As presented in the preceding paper (3), spectrophotometric and potentiometric evidence demonstrated that in 0.1 M sulfuric acid quinine is a dipolar cation with protons attached to the quinuclidine nitrogen and the nitrogen of the quinoline ring. The absorption spectrum of quinine changes progressively as the concentration of sul-
furic acid is increased from 0.1 to 17.5 M. The complete absorption spectra of quinine for the extremes of this range of concentrations of H$_2$SO$_4$ are shown in Fig. 2, and details of the progressive changes in a selected region are drawn to a larger scale in Fig. 3 in order to illustrate features of the changes which could not be shown on the smaller scale. The changes are reversible by dilution of the solutions with water. Again, a reversible proton exchange (with the methoxy group as possible acceptor) is sug-
gested, but the absence of true isosbestic points and the unusually wide range of concentrations of sulfuric acid in which the changes in absorption by quinine occur argue in favor of attributing the changes to a "medium effect," at least in part.

Quinine in 0.1 M H₂SO₄ is brilliantly fluorescent. From 0.1 to 3 m sulfuric acid there is a slight increase in the intensity of fluorescence of quinine, possibly due to a medium effect. The intensity of fluorescence of quinine remains the same in sulfuric acid from 3 to 7 M. From 7 to 18 M sulfuric acid there is a great and progressive diminution of fluorescence of quinine, the fluorescence being quite weak in 18 M acid. The mid-point of the change is 12 M sulfuric acid. The change is reversed by dilution of the solutions with water. This change in fluorescence of quinine, occurring concomitantly with the change in spectrophotometric absorption, suggests a reversible proton exchange, but a definite conclusion cannot be drawn on the basis of this evidence alone. Practical use can be made of the fact that the changes in fluorescence of quinine and pamaquine in concentrated aqueous solutions of sulfuric acid are in opposite directions. The data of Table II demonstrate that quinine can be determined specifically by measurement of its fluorescence in 0.1 M H₂SO₄, even in the presence of very large amounts of pamaquine (or SN-13276). On the other hand, pamaquine (or SN-13276) can be determined by measurement of its fluorescence in 18 M sulfuric acid with only minor interference from quinine when the two compounds are present in mixtures in nearly equal proportions. The interference from quinine becomes serious when it is present in much larger

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**Table II**

*Analysis of Mixtures of Quinine and Pamaquine by Measurement of Fluorescence in 0.1 M H₂SO₄ and Concentrated H₂SO₄ (18 M)*

<table>
<thead>
<tr>
<th>Quantity of pamaquine in mixture</th>
<th>Quantity of quinine in mixture</th>
<th>Concentration of H₂SO₄ in final solution</th>
<th>Quantity calculated from fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.3</td>
<td>0.1</td>
<td>Pamaquine: 1.27  Quinine: 1.37</td>
</tr>
<tr>
<td>1.26</td>
<td>1.3</td>
<td>0.1</td>
<td>Pamaquine: 1.31  Quinine: 1.28</td>
</tr>
<tr>
<td>2.62</td>
<td>1.3</td>
<td>0.1</td>
<td>Pamaquine: 1.29  Quinine: 1.26</td>
</tr>
<tr>
<td>5.04</td>
<td>1.3</td>
<td>18</td>
<td>Pamaquine: 1.24  Quinine:</td>
</tr>
<tr>
<td>1.26</td>
<td>0</td>
<td>18</td>
<td>Pamaquine: 1.37  Quinine: 1.52</td>
</tr>
<tr>
<td>1.26</td>
<td>1.3</td>
<td>18</td>
<td>Pamaquine: 1.52  Quinine: 1.86</td>
</tr>
<tr>
<td>1.26</td>
<td>2.6</td>
<td>18</td>
<td>Pamaquine: 1.86  Quinine: 2.63</td>
</tr>
<tr>
<td>1.26</td>
<td>5.2</td>
<td>18</td>
<td>Pamaquine: 2.63  Quinine:</td>
</tr>
<tr>
<td>1.26</td>
<td>13.0</td>
<td>18</td>
<td>Pamaquine:       Quinine:</td>
</tr>
</tbody>
</table>

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*6 The significance of the isosbestic point is discussed by Clark (4).*
amounts than pamaquine. However, even in the latter case pamaquine can be estimated from the fluorescence in 18 \text{m} \ H_2SO_4 by subtracting the fluorometric value which would be expected from the amount of quinine known to be present in the mixture from specific determination of quinine by measuring fluorescence in 0.1 \text{m} \ H_2SO_4. This is of practical value, inasmuch as quinine and pamaquine (or SN-13276) sometimes are administered together in the treatment of vivax malaria.

In the case of SN-3294, a large and progressive increase in fluorescence occurs in solutions of this compound in sulfuric acid from 7 to 18 \text{m}. This is the range in which great changes in spectrophotometric absorption by the compound occur (3, 5); the mid-points of the spectrophotometric and fluorometric changes practically coincide. These changes in absorption have been attributed to a reversible proton exchange involving the 4-amino group of SN-3294 as acceptor (3, 5). It appears likely that the change in fluorescence also is due to this proton exchange. In this case evidence for a proton exchange involving the methoxy group is lacking unless the small shift in the wave-lengths of the maxima of the absorption curves observed from approximately 7 to 14 \text{m} sulfuric acid is due to such a proton exchange rather than to a "medium effect," as suggested in the preceding paper (3).

**SUMMARY**

A fluorometric method is described for the determination of pamaquine, SN-13276, and SN-3294 in biological samples. The procedure also is applicable to mixtures of any one of these compounds with quinine. The method is based upon the fluorescence of these compounds in concentrated sulfuric acid. Spectrophotometric and fluorometric observations are recorded which suggest alternative explanations for the reversible changes occurring in solutions of these compounds in sulfuric acid.

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