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The Differential Determination of Mixtures of *p*-Aminosalicylic Acid and Sulphetrone or Sulphonamides in Body Fluids

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Since the introduction of *p*-aminosalicylic acid (PAS) to the chemotherapy of tuberculosis, several methods for its estimation have been described. These methods have depended on the presence of the free aryl amino group in the molecule, and have the disadvantage of a lack of specificity since free aryl amino groups are of frequent occurrence in synthetic drugs. Difficulties may therefore arise in estimating one in the presence of another. This can occur for instance in the combined use of PAS and diamino-diphenylsulphone derivatives, such as sulphetrone, (tetrasodium 4:4'-bis(γ -phenyl-*n*-propylamino) diphenylsulphone- $\alpha,\alpha',\gamma,\gamma'$ -tetrasulphonate), or PAS, and sulphonamide derivatives. All the drugs may be estimated singly by the standard method described by Bratton & Marshall (1939) of diazotizing and coupling with *N*-1-naphthylethylenediamine, and an examination of the visible absorption spectra of the coloured solutions (Fig. 1) shows that no differentiation is possible for PAS, sulphetrone, sulphanilamide and sulphathiazole, since the peak absorptions all lie in the same spectral area. This was also found to be true of other sulphonamides examined, namely, sulphadiazine, sulphaguanidine and sulphamezathine. To enable differentiation to be made, alternative methods of estimating the PAS alone have been examined. These have included the use of the reaction with ferric iron, coupling with *p*-dimethylaminobenzaldehyde, and coupling with diazonium salts of various amines in alkaline solution.

Reaction with ferric iron. PAS gives the characteristic salicylate reaction in neutral or faintly acid solution, producing a coloured complex with solutions of ferric salts. Sulphetrone and the sulphonamides do not give this reaction. It has been used for the estimation of PAS in urine (Venkatamaran, Venkatamaran & Lewis, 1948) and may be adapted to the estimation of high concentrations in blood

filtrates. Unfortunately the colour produced is not intense, making the method somewhat insensitive and of little value for general purposes.

Fig. 1. Visible absorption spectra after diazotizing and coupling with *N*-1-naphthylethylenediamine. Sulphetrone and PAS (1.0 mg./100 ml. of each), —; PAS (1.0 mg./100 ml.), —; sulphathiazole (0.2 mg./100 ml.), - - - -; sulphanilamide (0.2 mg./100 ml.), - · - · -; sulphetrone (1.0 mg./100 ml.), · · · · ·

Coupling with *p*-dimethylaminobenzaldehyde. *p*-Dimethylaminobenzaldehyde reacts with free aryl amino groups yielding characteristic lemon-yellow compounds. This has been applied by Venkatamaran *et al.* (1948) and others to the estimation of PAS. The sulphonamides give a similar reaction, and comparison of the visible absorption spectra of the coloured solutions at equivalent concentration shows the intensities of the colours to be approximately equal (Fig. 2). Sulphetrone also gives a colour with *p*-dimethylaminobenzaldehyde, but it is much less intense, and concentrations in the coloured solution of less than 0.2 mg./ml. do not differ significantly from the blank. At the dilutions

necessary for the estimation of PAS (generally 1/100), this would correspond to concentrations of sulphetrone in the blood (at least 20 mg./100 ml.) much higher than those normally encountered. Thus this method is satisfactory for the estimation of PAS in the presence of sulphetrone (Short, 1949), but not in the presence of sulphonamides.

Fig. 2. Visible absorption spectra after coupling with *p*-dimethylaminobenzaldehyde. PAS (0.2 mg./100 ml.), —; sulphathiazole (0.2 mg./100 ml.), - - - -; sulphanilamide (0.2 mg./100 ml.),; sulphetrone (0.5 mg./100 ml.), -

Fig. 3. Visible absorption spectra after coupling with diazo-*p*-nitraniline. PAS (0.8 mg./100 ml.), —; sulphathiazole (16.0 mg./100 ml.), - - - -; sulphanilamide (16.0 mg./100 ml.),; sulphaguanidine (16.0 mg./100 ml.),

Coupling with diazo solutions. PAS couples with solutions of the diazonium salts of various amines in alkaline solution to give coloured compounds of various intensities. Diazotized sulphanilic acid added to a solution of PAS followed by sodium hydroxide gives an orange, and diazotized *p*-nitraniline similarly gives a deep-red colour. Fig. 3 compares the visible absorption spectra for three

sulphonamides and PAS after coupling with diazotized *p*-nitraniline and making alkaline with sodium hydroxide. Solutions of 16 mg./100 ml. of the sulphonamides show absorptions which do not differ significantly from that of the blank solution. This method is suitable for general purposes and gives a valuable means of determining PAS, in the presence of sulphonamides, although its sensitivity is only two-fifths of that employing *p*-dimethylaminobenzaldehyde.

EXPERIMENTAL

Estimation of PAS in blood, urine and tissue filtrates by coupling with p-dimethylaminobenzaldehyde

The extension of the *p*-dimethylaminobenzaldehyde method used by Venkatamaran *et al.* (1948) to estimate PAS in blood and tissue filtrates has been studied in detail. The reaction is very sensitive to changes in pH and for maximum colour development the pH is critical, and should be between 2.0 and 2.2 (Table 1).

Table 1. *Effect of pH on the colour intensity of the product of PAS and p-dimethylaminobenzaldehyde*

(PAS 0.1 mg./100 ml.; Ilford filter no. 601.)

pH	Absorption (%)
3.72	18.5
3.35	22.9
3.00	25.5
2.50	29.5
2.15	31.9
2.06	32.2
1.87	30.5
1.55	26.8
1.42	20.2

A suitable filtrate is obtained by diluting with water and trichloroacetic acid so that the final concentration of the acid is 4.0% (w/v).

Reagents

Trichloroacetic acid. 12% (w/v) Aqueous solution.

***p*-Dimethylaminobenzaldehyde solution.** *p*-Dimethylaminobenzaldehyde (2.0 g.) dissolved in 100 ml. glacial acetic acid is added to 100 ml. 4M-sodium acetate. When 0.5 ml. of this reagent is added to 3.0 ml. water and 1.5 ml. trichloroacetic acid solution, the pH should lie between 2.0 and 2.2. If necessary, adjustment is made with either the *p*-dimethylaminobenzaldehyde solution in glacial acetic acid or 4M-sodium acetate.

Standard PAS solution. PAS is stable in solution only as the Na salt. The acid is purified by twice recrystallizing from ethanol and drying over P_2O_5 . A standard solution is prepared by suspending 100 mg. of the pure acid in a little water, warming gently with 4 drops of 20% (w/v) Na_2CO_3 solution and adjusting the volume to 100 ml. The standard solution contains 1 mg. PAS/ml.

Production of colour

Blood (0.1 ml.) is mixed with 5.9 ml. distilled water, 3.0 ml. of trichloroacetic acid is added, and after thorough mixing, the whole is filtered through a Whatman no. 5 filter

Table 2. *Recovery of PAS added to blood, estimated by coupling with p-dimethylaminobenzaldehyde*

(All results expressed as percentage recoveries are given, in this and subsequent tables, as the mean of six estimates $\pm$ S.E., with the range underneath in parentheses.)

PAS added (mg./100 ml.)	PAS recovered (%)		
	PAS alone	PAS with sulphetrone (5 mg./100 ml.)	PAS with sulphetrone (10 mg./100 ml.)
1.0	100.2 $\pm$ 0.7 (98.0-102.0)	100.4 $\pm$ 0.8 (98.0-104.0)	100.8 $\pm$ 2.5 (90.5-105.0)
5.0	98.3 $\pm$ 1.4 (96.0-102.0)	98.4 $\pm$ 1.3 (95.0-104.0)	102.4 $\pm$ 1.1 (99.6-106.0)
10.0	100.7 $\pm$ 0.8 (98.0-104.0)	100.7 $\pm$ 0.7 (98.0-103.5)	102.7 $\pm$ 1.5 (99.0-108.0)
20.0	100.5 $\pm$ 1.9 (96.0-107.0)	98.1 $\pm$ 1.1 (94.0-100.5)	99.6 $\pm$ 0.7 (98.0-102.5)

Table 3. *Recovery of PAS added to urine estimated by the p-dimethylaminobenzaldehyde method*

PAS added (mg./100 ml.)	PAS recovered (%)		
	PAS alone	PAS with sulphetrone (50 mg./100 ml.)	PAS with sulphetrone (100 mg./100 ml.)
10.0	97.5 $\pm$ 1.5 (90.0-102.0)	103.9 $\pm$ 2.8 (99.0-115.0)	101.8 $\pm$ 1.4 (98.0-107.0)
50.0	100.2 $\pm$ 0.7 (98.0-102.0)	100.3 $\pm$ 1.3 (98.0-106.0)	96.0 $\pm$ 1.8 (94.0-102.0)
100.0	100.3 $\pm$ 1.6 (94.0-104.0)	98.3 $\pm$ 1.1 (94.0-102.0)	104.4 $\pm$ 1.6 (100.0-108.0)
200.0	102.1 $\pm$ 1.5 (96.5-106.5)	95.8 $\pm$ 1.2 (92.0-98.5)	97.5 $\pm$ 1.2 (92.0-100.0)

paper. *p*-Dimethylaminobenzaldehyde reagent (0.5 ml.) is added to 4.5 ml. of the filtrate and the colour measured absorptiometrically, using an Ilford filter no. 601. (Maximum absorption is at 454 m μ ; see Fig. 2.) The colour may be measured 10 min. after the addition of the reagent and is stable for at least 3 hr.

The dilution of blood is 1/100. For PAS concentrations below 2.0 mg./100 ml., a dilution of 1/50 is better and for concentrations above 20 mg./100 ml. greater dilution is necessary, the volume of blood and distilled water being adjusted in each case so that the final concentration of trichloroacetic acid remains the same. PAS is bound to plasma proteins to a considerable extent. Way, Smith, Howie, Weiss & Swanson (1948) have shown that at plasma levels from 4 to 10 mg./100 ml. blood, approximately 50-60% is bound to plasma proteins. It is important that the original dilution of the blood is sufficient to free this combined portion of the drug. The dilution described (a minimum of 1/50) is ample for this purpose. With dilutions lower than 1/20 some of the drug is lost on the protein precipitate and recoveries are lower.

To estimate the drug in urine, preliminary dilution in distilled water is necessary. The diluted urine is then treated in the same way, trichloroacetic acid being added so that the final concentration is 4.0% (w/v).

Standard curves covering the final concentration range 0.0-2 mg./100 ml. in suitably diluted blood or urine are prepared from the standard solution in an exactly similar manner.

The concentration of PAS in tissue may be estimated by grinding 2 g. of the sample with washed silver sand in a glass

mortar, mixing with 10 ml. of distilled water and leaving to macerate for 1 hr. The contents of the mortar are then washed into a stoppered cylinder, the volume finally being adjusted to 60 ml. with distilled water. Trichloroacetic acid solution (30 ml.) is then added, the cylinder stoppered and well shaken. The mixture is filtered through a Whatman no. 5 filter paper and 4.5 ml. of the filtrate coloured in the usual way. The dilution of the tissue is 1/50. A further dilution of the filtrate with 4% trichloroacetic acid solution may be made if necessary.

Table 4. *Extent of darkening of p-dimethylaminobenzaldehyde reagent*

(The blank was prepared from 3.0 ml. distilled water, 1.5 ml. trichloroacetic acid and 0.5 ml. reagent. Ilford filter no. 601.)

Time after preparation of reagent (weeks)	Transmission of blank with reagent (%)		
	A	B	C
0	89.5	92.1	90.7
1.5	—	—	90.7
3	—	—	90.3
8	—	—	88.6
19	—	87.1	—
32	62.1	—	—

Tables 2 and 3 show the recoveries of PAS from blood and urine. Recoveries are expressed as percentages, and the values given are the mean of six determinations in each case.

PAS is equally well estimated at all concentrations used, the overall mean recovery being 99.9% in blood and 100% in urine.

A solution of *p*-dimethylaminobenzaldehyde darkens on keeping, but this effect is minimized by preparing the solution in acetate buffer as described. Table 4 shows the extent of darkening of various solutions over a period of 6 weeks. The reagent is suitable for absorptiometric work for at least 4 weeks and for direct visual comparison for longer periods if a similarly prepared blank is used.

Determination of PAS-sulphetrone mixtures

Sulphetrone is usually determined by diazotizing and coupling with *N*-1-naphthylethylenediamine (Brownlee, Green & Woodbine, 1948). PAS produces a similar colour, and the method has been applied to its estimation (Way *et al.* 1948; Newhouse & Klyne, 1949).

The reaction with PAS is less sensitive than that with sulphetrone (Fig. 1), but the colour produced by concentrations of the drug normally encountered in body fluids is sufficient to interfere with the estimation of sulphetrone. A differential method for estimating drugs by using the *p*-dimethylaminobenzaldehyde and naphthylethylenediamine reactions has been studied.

Sulphetrone gives only a faint colour with *p*-dimethylaminobenzaldehyde (Fig. 2), and at the dilutions generally employed PAS may be estimated accurately in the presence of the sulphetrone. Tables 2 and 3 give the results of estimation of PAS added to blood and urine containing 5 and 10 mg./100 ml. of sulphetrone, and 50 and 100 mg./100 ml. PAS respectively. Recoveries, expressed as percentages, are the mean of six determinations in each case. PAS is equally well estimated at all concentrations and in all the mixtures employed, overall recoveries being within $100 \pm 0.25\%$ in blood and in urine.

The 'total diazotizable substances' (sulphetrone and PAS) are next determined by the method of Brownlee *et al.* (1948). Standard curves for sulphetrone of final concentration range 0–1 mg./100 ml. (Fig. 4, curve A) and PAS of final concentration range 0–2 mg./100 ml. (Fig. 4, curve B) are prepared similarly from standard solutions in corresponding dilutions of blood and urine.

Calculation of results. The 'total diazotizable concentration' in terms of sulphetrone is determined by reading the percentage absorption from curve A. The absorption given by the concentration of PAS in the filtrate (independently determined by the *p*-dimethylaminobenzaldehyde method) is read from curve B. The concentration of sulphetrone equivalent to this absorption is then determined from curve A. This gives the correction for the colour due to the PAS and subtraction of this from the 'total diazotizable concentration' gives the actual concentration of sulphetrone in the filtrate. Typical standard curves A and B and a specimen protocol of the results are given in Fig. 4.

Recoveries obtained by this method when sulphetrone was added to blood and urine containing varying concentrations of PAS, are given in Tables 5 and 6.

Satisfactory recoveries from urine were obtained in all the mixtures employed, the overall mean recovery being 100.1%. The results with blood show a greater variation, the mean percentage recovery with the lower concentration of sulphetrone (5 mg./100 ml.) of 100.0% indicates complete recovery of the drug. The corresponding figure for the higher sulphetrone concentration (10 mg./100 ml.) is 107.1%;

although the difference between this figure and 100 is statistically significant, it is not sufficiently great to be of clinical importance, especially as such high blood concentrations are to be avoided in clinical practice. It may be that the high sulphetrone concentration prevents complete removal of the PAS from the plasma protein, resulting in its low estimate. Support is lent to this hypothesis by the fact that mixtures of higher concentrations in urine do not give these high recoveries.

Fig. 4. Standard curves for the estimation of sulphetrone (curve A) and PAS (curve B) by diazotizing and coupling with *N*-1-naphthylethylenediamine. Ilford filter no. 605. An absorption of 35.9% (a) corresponds to a 'total diazotizable concentration' in terms of sulphetrone of 0.5 mg./100 ml. (b) see curve A. Let the concentration of PAS in the final filtrate, calculated after independent determination of the concentration of PAS in the sample by coupling with *p*-dimethylaminobenzaldehyde, be 0.8 mg./100 ml. (c) see curve B, the absorption corresponding to 0.8 mg./100 ml. PAS is 15.2% (d), and this percentage absorption corresponds to 0.15 mg. sulphetrone/100 ml. and the actual concentration of sulphetrone in the filtrate is given by subtraction of this value from the 'total diazotizable concentration' ($0.5 - 0.15 = 0.35$ mg. sulphetrone/100 ml.). Correction for the dilution then gives the concentration of sulphetrone in the sample.

Estimation of PAS by coupling with diazotized-*p*-nitraniline

Various methods of estimation depending on the coupling of PAS with diazo solutions have been described. The use of diazotized *p*-nitraniline is described by Tennent & Leland (1949), who employed a differential method of coupling in the presence of pyridine on the one hand and extracting the dye with pyridine on the other to eliminate interfering colours, and Street (1949), who diazotized both the PAS and *p*-nitraniline and then made alkaline with NaOH.

In attempts to apply these methods to mixtures containing PAS and sulphonamides, it was found that the colour pro-

Table 5. Recoveries of sulphetrone added to blood in the presence of PAS

Sulphetrone added (mg./100 ml.)	PAS concentration (mg./100 ml.)			
	1.0	5.0	10.0	20.0
	Sulphetrone recovered (%)			
5.0	99.2±1.7 (94.0-103.6)	98.8±1.9 (90.0-103.0)	103.0±1.0 (101.0-107.0)	99.0±1.9 (94.0-104.0)
10.0	105.5±0.8 (102.0-107.0)	108.5±1.9 (103.0-112.0)	106.8±1.3 (104.0-112.0)	107.5±2.2 (103.0-115.0)

Table 6. Recoveries of sulphetrone added to urine in the presence of PAS

Sulphetrone added (mg./100 ml.)	PAS concentration (mg/100 ml.)				
	1.0	5.0	10.0	20.0	200.0
	Sulphetrone recovered (%)				
5	94.9±0.7 (93.2-98.0)	100.6±1.0 (98.0-103.8)	97.8±1.6 (90.0-100.0)	103.3±3.5 (90.0-112.0)	—
10	96.5±0.8 (95.2-99.7)	95.2±0.8* (93.3-101.2)	101.6±1.8 (94.8-108.2)	106.5±3.5 (104.0-120.0)	—
100	—	—	—	—	96.8±1.2* (94.0-100.0)
150	—	—	—	—	105.6±1.8† (101.0-110.0)
200	—	—	—	—	101.8±0.9 (98.5-105.0)

* Four estimates.

† Five estimates.

Table 7. Recoveries of PAS in the presence of various sulphonamides

(Concentration of sulphonamide 100 mg./100 ml. PAS estimated by the diazotized *p*-nitraniline method.)

PAS added (mg./100 ml.)	PAS recovered (%)		
	PAS with sulphanilamide	PAS with sulphadiazine	PAS with sulphaguanidine
20.0	101.3±2.9 (91.5-106.0)	102.7±1.5 (99.0-107.0)	102.2±2.2 (95.0-110.5)
50.0	99.2±1.7 (91.2-104.0)	95.4±1.3 (90.4-100.0)	98.8±2.5 (92.0-108.0)
100.0	96.9±2.7 (92.5-110.0)	96.3±2.7 (90.0-103.5)	96.1±0.9 (94.0-100.0)

duced by the latter was due to excess nitrite added with the diazotized *p*-nitraniline solution. This diazotized the sulphonamide which then coupled with the reagent to give a coloured complex. If, however, the excess nitrite was removed from the diazotized *p*-nitraniline solution, no significant colour was produced in solutions containing at least twenty times the concentration of PAS suitable for measurement (Fig. 3).

Reagents

Trichloroacetic acid. 12% (w/v) Aqueous solution.

Sodium hydroxide. 2*N*.

Sodium nitrite. 0.3% (w/v) Aqueous solution. This should be freshly prepared each week.

Ammonium sulphamate. 1.5% (w/v) Aqueous solution.

p-Nitraniline. 0.05% (w/v) Solution in 0.1*N*-HCl.

Diazotized p-nitraniline solution. This should be freshly prepared by adding to 10.0 ml. *p*-nitraniline solution 1 ml. NaNO₂ solution, allowing to stand for 3 min. after thorough

mixing, and then adding 0.3 ml. ammonium sulphamate solution. The reagent should be left for at least 5 min. after the addition of the sulphamate before use. It is stable for about 3 hr.

Production of colour

Blood (0.4 ml.) is added to 3.6 ml. distilled water and 3.0 ml. of trichloroacetic acid added. After thorough mixing, the protein precipitate is removed by filtering through a Whatman no. 5 filter paper. Diazotized *p*-nitraniline solution (0.5 ml.) is added to 3.5 ml. of the filtrate, the mixture allowed to stand for 3 min., and 1.0 ml. of NaOH solution then added. A deep-red colour develops immediately and, after 15 min., it is measured absorptometrically, using an Ilford filter no. 604. (Maximum absorption is at 524 mμ.; see Fig. 3.)

The dilution of the blood is 1/25. For concentrations above 15 mg./100 ml. greater dilution is necessary, the volume of distilled water and blood being adjusted so that the ratio of

trichloroacetic acid, diazotized *p*-nitraniline and NaOH solutions remains the same.

To estimate the drug in urine, preliminary dilution in distilled water may be necessary. The diluted urine is then treated in the same way, the ratio of the reagents being kept as above.

Corresponding standard curves in blood and urine of final concentration range 0–1.0 mg./100 ml. are prepared similarly, using dilutions of the standard solution.

The recoveries of PAS estimated by this method in the presence of various sulphonamides (Table 7) show that it is equally efficient in the three mixtures.

Determination of PAS-sulphonamide mixtures

A differential method, similar to that described for the estimation of mixtures of PAS and sulphetrone, may be applied to the determination of mixtures of PAS and the sulphonamides.

The 'total diazotizable substance' is determined by the naphthylethylenediamine method, the PAS is estimated independently by coupling with diazotized *p*-nitraniline, and the correction for the PAS contribution then calculated as described on p. 304.

Recoveries of 91–108% were obtained in the estimation of mixtures of PAS and sulphanilamide, sulphadiazine or sulphaguanidine.

SUMMARY

1. Methods for determining *p*-aminosalicylic acid (PAS) in body fluids are reviewed.

2. Convenient methods for estimating *p*-aminosalicylic acid in the presence of sulphetrone and other sulphone derivatives, using *p*-dimethylaminobenzaldehyde, and in the presence of sulphonamides by coupling with diazotized *p*-nitraniline, are described.

3. A differential method for the determination of mixtures of *p*-aminosalicylic acid and sulphetrone is described.

4. A similar method for the determination of mixtures of *p*-aminosalicylic acid and sulphonamide drugs is described.

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Paper Chromatography of Porphyrins: some Hitherto Unrecognized Porphyrins and Further Notes on the Method

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Filter-paper chromatography, using a lutidine-water system, has been applied to the separation of the porphyrins by Nicholas & Rimington (1949). The R_F values of free porphyrins are inversely proportional to the number of carboxyl groups in the molecule; hence with the aid of a reference graph one may deduce from the R_F values obtained in any paper chromatographic analysis of a mixture of porphyrins what classes of porphyrins are present, e.g. dicarboxylic porphyrins, coproporphyrins or uroporphyrins.

The technique has proved of value in demonstrating the presence in normal urine of small quantities of uroporphyrin together with coproporphyrin (Nicholas & Rimington, 1949), and also in

identifying the porphyrins of the 'pearl shells' *Pteria (Pinctada) radiata* and *Pteria vulgaris* as uroporphyrin and coproporphyrin (Nicholas & Comfort, 1949). No support was obtained from paper chromatograms for the existence in marine molluscan shells of the pentacarboxylic porphyrin, termed 'conchoporphyrin' by Fischer & Hofmann (1937).

When the total urinary porphyrins of some cases of acute porphyria were examined by this method, it was discovered that in addition to known porphyrins containing two, four or eight carboxyl functions, there were frequently present appreciable quantities of porphyrins giving discrete fluorescent spots, the R_F values of which indicated groups with three,