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Colorimetric analysis of immunogenic impurities in acetylsalicylic acid

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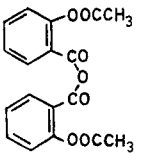
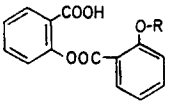
A rapid and convenient colorimetric method is described for the quantitative determination of the immunogenic impurities in acetylsalicylic acid, acetylsalicylsalicylic acid and acetylsalicylic anhydride. The method involves initial aminolysis of the compounds by ammonia to give salicylamide and subsequent coupling of this with 4-amino-phenazone in the presence of an oxidizing agent. In conjunction with a previously described method for analysis of the anhydride the method allows a specific determination of acetylsalicylsalicylic acid in concentrations down to 0·005%. Applying the methods to 15 different commercial acetylsalicylic acid samples and formulations, acetylsalicylic anhydride and acetylsalicylsalicylic acid were found to be present in amounts ranging from 0·001 to 0·024% and from 0·006 to 0·58% (w/w), respectively.

Impurities in acetylsalicylic acid rather than the drug substance itself are held responsible for the appearance of anti-salicyloyl antibodies in patients treated with acetylsalicylic acid (De Weck, 1971; Lazary, Toffler & De Weck, 1972; Bundgaard, 1974; Bundgaard & De Weck, 1975).

Substances reported as minor contaminants of acetylsalicylic acid include acetylsalicylic anhydride (I) De Weck, 1971; Bundgaard & Bundgaard, 1973) and acetylsalicylsalicylic acid (II, R = Ac) (Patel, Perrin & Windheuser, 1972). In addition, other possible impurities such as salicylsalicylic acid (II, R = H) itself and *cis*-disalicylide (III), have been reported to show similar immunological effects (Bundgaard & De Weck, 1975; Schlumberger, 1975).



I



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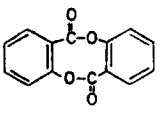


III

Bundgaard & Bundgaard (1973) have previously described a spectrophotometric method for the determination of I in acetylsalicylic acid. The present paper describes an assay specific for acetylsalicylsalicylic acid (II, R = Ac).

MATERIALS AND METHODS

Reagents and chemicals were prepared as follows: *4-Aminophenazone solution*—0·5 g was dissolved in 100 ml of water. This solution remains stable for 3 days. *Potassium ferricyanide solution*—2·0 g was dissolved in 100 ml of water. This solution remains stable for 3 days. *4M ammonia solution pH 10·4*—conc. ammonia (27–30% w/w of NH_3) was diluted with water and concentrated hydrochloric acid to the specified molarity and pH 10·4 ± 0·1 (measured at 20–25°).

Acetylsalicylic anhydride and acetylsalicylsalicylic acid were prepared as previously described (Bundgaard & Bundgaard, 1973; Bundgaard, 1974). Salicylsalicylic acid (Fluka AG) was recrystallized from benzene. *Cis*-disalicylide was prepared as described by Anschütz & Riepenkröger (1924), m.p. 233° (from chloroform), lit. m.p. 234° (Baker, Ollis & Zeally, 1951). All other chemicals and solvents used were of reagent grade quality. Acetylsalicylic acid was recrystallized four times from 95% ethanol.

Analytical procedure

Transfer an accurately weighed quantity of acetylsalicylic acid (100 mg ± 10%), or of powdered tablet equivalent to this weight, to a screw-capped test tube, and add 4 M ammonia solution (2·0 ml). After dissolution of the acetylsalicylic acid allow the tube and contents to stand either at 40° for

15 min, or at 20–25° for 1 h. When tube and contents are at room temperature add about (see note below) 2 ml of 2 M hydrochloric acid and 6 ml of water. Then add the 4-aminophenazone solution (0·50 ml), mix well and add potassium ferricyanide solution (0·50 ml), and mix again. Within 5 min add n-butanol (10·00 ml), shake thoroughly and allow the phases to separate. Transfer a portion of the upper n-butanol layer to a tube and centrifuge for 2 min. Measure the absorbance of the clear n-butanol phase in a 1 cm cell at 505 nm, using n-butanol as the reference solution within 30 min. Determine the content of acetylsalicylsalicylic acid in the acetylsalicylic acid sample by reference to a standard curve, or by calculating it from the formula:

$$\%\ \text{acetylsalicylsalicylic acid} = \frac{(A - 0{\cdot}030) \times 10}{600 \times W}$$

where A is the measured absorbance and W is the weight of the acetylsalicylic acid sample (g); the factor 600 is A (1%, 1 cm) as determined for pure acetylsalicylsalicylic acid. The factor 0·030 is a correction for the absorbance of salicylic acid dye, produced from the acetylsalicylic acid (0·1 g) during the assay conditions (see Discussion).

Note: The amount of 2 M hydrochloric acid used should be such that pH of the solution before colour development is within the range 8·1–8·4.

For acetylsalicylic acid samples with a content of acetylsalicylsalicylic acid exceeding 0·2% a reduced amount of sample should be taken. This affects both the amount of hydrochloric acid necessary to adjust the pH to 8·1–8·4 and the correction factor in the formula. If, for example, only 50 mg of acetylsalicylic acid is subjected to analysis, the factor will be 0·015.

In the few cases where acetylsalicylic acid samples contain acetylsalicylic anhydride in amounts exceeding a fifth of the amount of acetylsalicylsalicylic acid, the content of the latter is calculated by the following formula:

$$\%\ \text{acetylsalicylsalicylic acid} = \frac{[(A - 0{\cdot}030 - (\%\text{ASAN} \times W \times 28)] \times 10}{600 \times W}$$

where % ASAN means the percentage (w/w) content of acetylsalicylic anhydride in the acid sample (Bundgaard & Bundgaard, 1973). The factor 28 is a tenth of A (1%, 1 cm) for the anhydride.

Preparation of standard curves

Prepare solutions of acetylsalicylsalicylic acid in chloroform and evaporate portions corresponding to 10–150 µl of acetylsalicylsalicylic acid under a mild air current in test tubes. Transfer to each tube 100 mg of recrystallized acetylsalicylic acid and continue as described in the analytical procedure. From the slope of the straight line produced by plotting absorbance against concentration of acetylsalicylsalicylic acid the molar absorbance and A (1%, 1 cm) are $1{\cdot}8 \times 10^4$ and 600, respectively. The intercept of the line corresponds to an absorbance of 0·030 arising from salicylic acid dye.

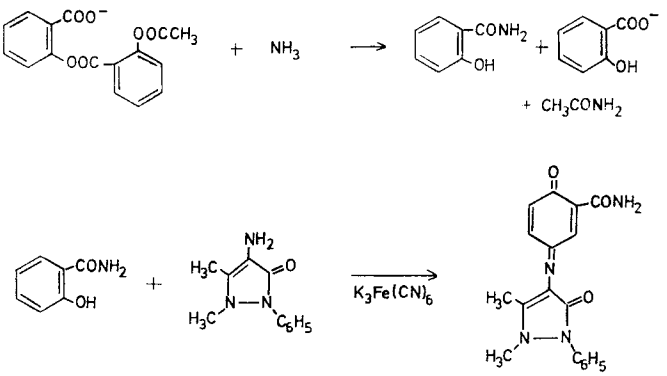
For acetylsalicylic anhydride the molar absorptivity and A (1%, 1 cm) are $9{\cdot}5 \times 10^3$ and 280, respectively.

RESULTS AND DISCUSSION

The determination of acetylsalicylsalicylic acid is based on initial aminolysis to give salicylamide (cf. Bundgaard (1974)) and subsequent colorimetric determination by coupling with 4-aminophenazone, cf. Mohler & Jacob (1957); Johnson & Savidge (1958); Svobodova & Gasparic (1971) (Scheme 1).

Under the assay conditions acetylsalicylic acid itself is transformed quantitatively to salicylic acid and this reacts with 4-aminophenazone to form a measurable colour. Such interference by salicylic

Scheme 1



acid can be eliminated almost completely by extraction of the dye produced from salicylamide with n-butanol. At pH 8 the salicylic acid dye, in contrast to the salicylamide dye, is ionized and therefore water-soluble. The amount of salicylic acid dye being extracted into the n-butanol corresponded to an absorbance of 0·030 (correction factor) when 100 mg of purified acetylsalicylic acid or an equivalent amount of purified salicylic acid (76·7 mg) or sodium salicylate (88·9 mg) were used.

Fig. 1A shows that the aminolysis of acetylsalicylsalicylic acid is complete after 15 min at 40° or after 1 h at 23°. The conversion of acetylsalicylsalicylic acid into salicylamide proceeds quantitatively as shown by identity between the molar absorptivities of acetylsalicylsalicylic acid and of the equivalent amount of salicylamide added separately to pure acetylsalicylic acid samples, and assayed as described.

Specificity of the method

In principle the method is capable of determining any impurity in acetylsalicylic acid that will react with ammonia to yield salicylamide (and accordingly is to be regarded as an immunogenic substance), *e.g.* acetylsalicylsalicylic acid, salicylsalicylic acid, acetylsalicylic anhydride, and *cis*-disalicylide. Whereas salicylsalicylic acid behaved exactly like acetylsalicylsalicylic acid (cf. Bundgaard, 1974), the anhydride and *cis*-disalicylide, on a molar concentration basis, produced a less intense colour than acetylsalicylsalicylic acid. The molar absorptivities of the anhydride and disalicylide are $9{\cdot}5 \times 10^3$ and $12{\cdot}2 \times 10^3$, respectively. Such lower colour yield arises because with ammonia the anhydride gives *N*-acetylsalicylamide, while the disalicylide gives disalicylimide, in addition to salicylamide. These products have a lower molar absorptivity than salicylamide itself after coupling with 4-aminophenazone. Both anhydride and disalicylide react faster with ammonia than acetylsalicylsalicylic acid.

A specific method for the determination of acetylsalicylic anhydride is available (Bundgaard & Bundgaard, 1973) and the contribution of this substance to the measured absorbance can therefore be obtained. Most acetylsalicylic acid preparations have a lower content of anhydride than of acetylsalicylsalicylic acid (see Table 1) and this contribution can normally be neglected.

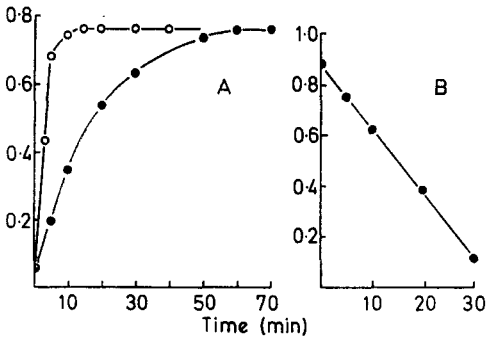


FIG. 1. (A) Time course of formation of salicylamide by ammonolysis of acetylsalicylsalicylic acid at 23° (●) and 40° (○). The reaction solutions consist of 100 mg of purified acetylsalicylic acid and 127 μg of acetylsalicylsalicylic acid in 2·00 ml of 4 M ammonia solution pH 10·4. Salicylamide is determined colorimetrically with 4-aminophenazone. (B) First-order plot for the formation of salicylamide from acetylsalicylsalicylic acid at 23°. Ordinates: A—absorbance (505 nm); B—log $(A_\infty - A_t)+1$.

Table 1. *Results of analysis for acetylsalicylsalicylic acid and acetylsalicylic anhydride in 15 different acetylsalicylic acid samples and formulations commercially available in Denmark.*

Sample	Acetylsalicylic anhydride (%)	Acetylsalicylsalicylic acid (%)
A	0·024	0·12
B	0·0016	0·30
C	0·0012	0·025
D	0·0014	0·006
E	0·0029	0·024
F	0·0024	0·015
G	0·0078	0·20
H	0·0066	0·027
I	0·0028	0·23
J	0·014	0·083
Tablet K	0·020	0·045
Tablet L	0·0081	0·37
Tablet M	0·0092	0·58
Tablet N	0·019	0·38
Tablet O	0·011	0·51

It is possible to distinguish between salicylsalicylic acid and acetylsalicylsalicylic acid by using the following modification of the method. The acetylsalicylic acid sample is dissolved in borate buffer pH 8·5 at room temperature and the oxidizing 4-aminophenazone reagent is added. Under these conditions the amounts of salicylic acid formed are low and do not give any colour. In contrast to acetylsalicylsalicylic acid, salicylic acid possesses a free phenolic group and thus forms a dye with the reagents. No salicylsalicylic acid has been detected in any of our various acetylsalicylic acid preparations.

Cis-disalicylide was present in a very few acetylsalicylic acid preparations and in amounts less than 0·005% (Bundgaard, to be published).

Precision and sensitivity of the method. Ten determinations were made on a purified acetylsalicylic acid sample with a given amount of acetylsalicylsalicylic acid and acetylsalicylic anhydride added as described under "standard curve". The relative standard deviations were 1·8% and 2·0%, respectively. The method permits determination of 0·005% of acetylsalicylsalicylic acid.

Analysis of commercial acetylsalicylic acid samples and formulations. Ten different samples of crystalline acetylsalicylic acid and five different tablet formulations marketed in Denmark were assayed for their content of acetylsalicylic anhydride by the method of Bundgaard & Bundgaard (1973) and for their content of acetylsalicylsalicylic acid by the present method (Table 1). All contain quantifiable concentrations of both impurities, the concentrations of acetylsalicylsalicylic acid being considerably higher than those of anhydride in most preparations. Salicylsalicylic acid was not found in detectable amounts (<0·005%). Tablet formulations are particularly contaminated with acetylsalicylsalicylic acid. The common tablet excipients have been shown to produce no interference in the method. In addition, when the tablets were rapidly extracted by tetrahydrofuran and an aliquot of the filtered solution corresponding to 50 mg of acetylsalicylic acid evaporated and analysed by the method identical results (within ±5%) were obtained.

Kinetic treatment of the reaction between acetylsalicylsalicylic acid and ammonia to produce salicylamide can be used to provide further evidence for the identification of acetylsalicylsalicylic acid as an impurity in the preparations. Fig. 1B shows that the reaction follows apparent first-order kinetics under the assay conditions with a rate constant of 0·059 min^{-1} at 23°. Analysis of several of the acetylsalicylic acid preparations (in case of tablets a prior extraction by tetrahydrofuran was done) in this manner gave apparent first-order rate constants identical (within ±10%) to the value obtained with authentic acetylsalicylsalicylic acid.

We have found that the gas chromatographic method of Patel & others (1972), for the analysis of acetylsalicylsalicylic acid in acetylsalicylic acid was of low precision, with a sensitivity of not less than 0·03–0·04%.

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