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Spectrophotometric determination of microgram quantities of acetylsalicylic anhydride in acetylsalicylic acid

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A rapid and convenient method for the determination of an immunogenic impurity in acetylsalicylic acid, acetylsalicylic anhydride, is described. The method is based on the spectrophotometric measurement at 362 nm of 4-benzylidene-2-phenyloxazolin-5-one, formed in quantitative yield by allowing acetylsalicylic anhydride, pre-extracted from the acetylsalicylic acid by a benzene-aqueous phosphate buffer system, to react with a mixture of pyridine and sodium α-benzamidocinnamate in ethanol for 30 min at 20°. The method is reproducible and permits determination of 0·0005% of acetylsalicylic anhydride in acetylsalicylic acid. Applying the method to nine different commercial acetylsalicylic acid preparations, acetylsalicylic anhydride was found to be present in amounts ranging from 0·0012 to 0·024%.

It has recently been suggested that allergic reactions to acetylsalicylic acid may be due to it containing an impurity, acetylsalicylic anhydride (De Weck, 1971). By thin-layer chromatography De Weck has detected acetylsalicylic anhydride in most commercial acetylsalicylic acid preparations in amounts considered to lie within the range of 0·01 – 0·1%. The anhydride was found to be a highly immunogenic substance capable of inducing the formation of acetylsalicyloyl-specific antibodies and of contact hypersensitivity in guinea-pigs and rabbits. De Weck has also found that commercial acetylsalicylic acid preparations varied greatly in their capacity for inducing anti-acetylsalicylic anhydride hypersensitivity under standardized conditions in guinea-pigs, but the lack of a method for the quantitative determination of the minute amounts of the anhydride present in preparations of the acid prevented the determination of whether the immunogenicity of the acetylsalicylic acid preparations correlated quantitatively with the amounts of the anhydride impurities present in the preparations. If such a correlation existed and could be established, it would be strong evidence for the proposal that the immunogenic effect of acetylsalicylic acid is due to an acetylsalicylic anhydride impurity and not to the acid itself.

Owing to the definite immunological effect of acetylsalicylic anhydride, controls on the level of it present in preparations of the acid must be set up in the future and for this reason too a sensitive analytical method is required.

We have developed a rapid and convenient spectrophotometric method for the quantitative determination of 0·0005% acetylsalicylic anhydride present in acetylsalicylic acid. The method can also be used for the determination of other carboxylic anhydrides, but this will be described elsewhere.

MATERIALS AND METHODS

Apparatus. A Zeiss PMQ II spectrophotometer and a Radiometer model PHM 26 pH meter were used.

Materials and reagents

Synthesis of acetylsalicylic anhydride. To a solution of 7·2 g (0·04 mol) of acetylsalicylic acid in 75 ml of tetrahydrofuran was added 4·12 g (0·02 mol) of *NN*-dicyclohexylcarbodi-imide dissolved in 30 ml of tetrahydrofuran, and the mixture was set aside overnight at room temperature. Dicyclohexylurea was filtered off and the filtrate evaporated in a vacuum. The residue was dissolved in 6 ml of ethanol by heating on a steam bath and then cooled, giving crystalline acetylsalicylic anhydride (5·0 g), m.p. 85–86° (from ethanol); melting points reported in the literature lie in the range 83–86°. The product had ultraviolet and infrared spectra in agreement with acetylsalicylic anhydride.

Synthesis of sodium α-benzamidocinnamate. 4-benzylidene-2-phenyloxazolin-5-one was prepared in 74% yield by the condensation of benzaldehyde with hippuric acid in acetic anhydride containing anhydrous sodium acetate according to the method of Gillespie & Snyder (1943), m.p. 166–167° (from benzene), lit. m.p. 167–168°. α-Benzamidocinnamic acid was prepared by alkaline hydrolysis of the 4-benzylidene-2-phenyloxazolin-5-one as described by Gillespie & Snyder (1943), m.p. 230–231° (from ethanol), lit. m.p. between the limits 224° and 236°. The sodium salt of the α-benzamidocinnamic acid was finally obtained by addition of 80 ml of ethanol to a solution of α-benzamidocinnamic acid (1·0 g) in 2N sodium hydroxide (1·9 ml). After standing overnight at 0° the fine white crystals of sodium α-benzamidocinnamate which had formed were filtered and dried (1·05 g).

All other chemicals used were of reagent grade or of Ph.Nord. 63 quality. The commercial samples of acetylsalicylic acid conformed in all respects to the requirements of the Pharmacopoeia Nordica 1963.

α-Benzamidocinnamate-pyridine reagent: dissolve 0·15 g of sodium α-benzamidocinnamate in 5 ml of water, add 10·0 ml of pyridine and dilute to 500 ml with ethanol.

0·5 M phosphate buffer solution pH 8·0: potassium dihydrogen phosphate (34 g), 2N sodium hydroxide (120 ml) and water (to 500 ml).

0·5 M phosphate buffer solution pH 11·3: potassium dihydrogen phosphate (34 g), 2N sodium hydroxide (190 ml) and water (to 500 ml).

Analytical procedure

Transfer an accurately weighed quantity of acetylsalicylic acid (500 mg ± 10%) to a test tube, and add 2000 μl of benzene and then 10·0 ml of the phosphate solution pH 11·3. Stopper the test tube and shake it manually or mechanically for 10 min. Allow the phases to separate and transfer with a micropipette an aliquot (50–500 μl) of the benzene layer into a small glass tube. Evaporate the benzene under a mild air current (about two min are required to evaporate 500 μl of benzene) and add 2000 μl of the α-benzamidocinnamate-pyridine reagent. Stopper the glass tube and shake it to ensure complete dissolution of the residue of the evaporation. Allow the solution to stand for 30 min at room temperature (20–25°) and within 2 h measure the extinction of the solution in a 1 cm cell at 362 nm, using the α-benzamidocinnamate-pyridine reagent as

reference solution. Determine the content of acetylsalicylic anhydride in the acetylsalicylic acid sample by reference to a standard curve, or calculate it by the formula:

$$\% \text{ acetylsalicylic anhydride} = \frac{E \times 4}{1040 \times \mathrm{P} \times \mathrm{W}}$$

where E is the measured extinction, P is the portion (in ml) of the benzene phase evaporated and W is the weight of the acetylsalicylic acid sample (in g); 1040 is $E(1\%, 1\text{ cm})$ as determined for acetylsalicylic anhydride.

The amount of benzene to be evaporated depends on the content of acetylsalicylic anhydride in the acetylsalicylic acid sample. For example, an absorbance value of 0·3 corresponds to a content of anhydride in the acetylsalicylic acid of 0·005% if 500 μl of benzene are evaporated and 0·05% if 50 μl are evaporated.

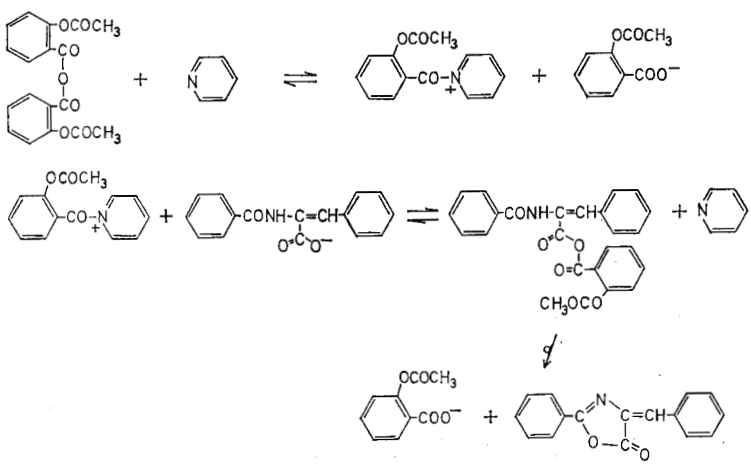
Preparation of standard curve

Prepare solutions of acetylsalicylic anhydride in benzene at concentrations within the range of 2–30 μg ml^{-1}. Mix 2000 μl of these solutions with 10·0 ml of the phosphate buffer pH 8·0 and continue as described in the analytical procedure transferring 500 μl of the benzene phase to a glass tube for evaporation. A straight-line relation between extinction and concentration of acetylsalicylic anhydride within the cited concentration range was observed, indicating adherence to Beer's law. From the slope of the line, the molar extinction and E (1%, 1 cm) were determined to be $3{\cdot}56 \times 10^4$ and 1040, respectively.

RESULTS AND DISCUSSION

Principle of the method

The quantitative determination of acetylsalicylic anhydride is based on the measurement of the extinction of the strong ultraviolet-absorbing 4-benzylidene-2-phenyloxazolin-5-one, formed by an intramolecular rearrangement of the mixed anhydride of acetylsalicylic acid and α-benzamidocinnamic acid (Scheme 1). It has long been known (e.g. Cornforth, 1949; Baltazzi, 1955) that strongly activated derivatives such as acid chlorides or anhydrides of α-acylamino acids (e.g. α-benzamidocinnamic acid) are



quite unstable and spontaneously undergo rearrangements into the corresponding oxazolones. But the formation of the mixed anhydride proceeds at too slow a rate without a catalyst, and it is therefore necessary to accelerate the reaction, for which purpose pyridine was found efficient providing the concentration was below 3%: 2% concentration was adopted. As shown in Scheme 1 the mode of the pyridine catalysis is thought to be a nucleophilic catalysis mechanism with the intermediate formation of a highly reactive acetylsalicyloylpyridinium ion. At pyridine concentrations greater than 3% a greater amount than the theoretical value of the 4-benyzlidene-2-phenyloxazolin-5-one was formed (Fig. 1). This was due to a reaction between

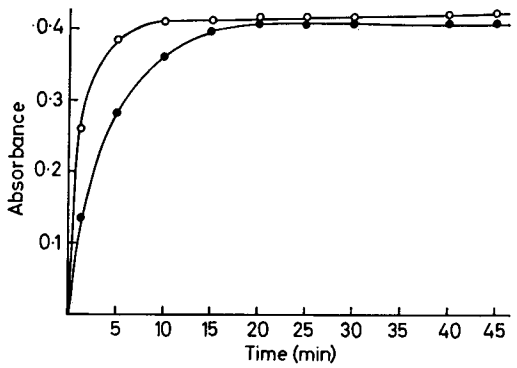


FIG. 1. The effect of pyridine concentration—2% v/v (●) and 10% v/v (○)—on time course of formation of 4-benzylidene-2-phenyloxazolin-5-one at 22°. The reaction solution consists of acetylsalicylic anhydride (3·9 μg ml^{-1}), sodium α-benzamidocinnamate (0·03%), and pyridine in ethanol 99% v/v. Absorbance is measured at 362 nm.

pyridine and the two moles of acetylsalicylate, arising from the acetylsalicylic anhydride (Scheme 1). The product from this reaction, acetylpyridinium ion, behaves as an acetylsalicyloylpyridinium ion and also reacts with the α-benzamidocinnamate anion to produce the oxazolone.

Effect of solvent. Of the solvents examined (dioxane, dimethylformamide, acetonitrile, acetone, benzene, methanol, ethanol, propanol, and water) ethanol was found to be the best. In the other solvents the rate of the oxazolone formation was either very slow (e.g. in dioxane), the yield of formed oxazolone was only about 50% (in water), or the interference of the acetylsalicylate produced from acetylsalicylic anhydride was pronounced (e.g. in acetonitrile and acetone). A content of water in the ethanol in concentrations up to 5% has no effect.

Effect of sodium α-benzamidocinnamate concentration. As it appears from the reaction scheme the quantitative determination of acetylsalicylic anhydride is conditional on an excess (on the molar basis) of α-benzamidocinnamate over the anhydride. This condition is fulfilled with a concentration of sodium α-benzamidocinnamate of 0·03% w/v ($\sim 1{\cdot}4 \times 10^{-3}$M). With α-benzamidocinnamate in excess over the anhydride, the rate as well as the extent of oxazolone formation was independent of variations of the concentration of sodium α-benzamidocinnamate.

With a reaction solution consisting of sodium α-benzamidocinnamate (0·03%), pyridine (2% v/v) and water (1% v/v) in ethanol, the acetylsalicylic anhydride-induced formation of the 4-benzylidene-2-phenyloxazolin-5-one is almost quantitative. Based on a molar extinction of $3{\cdot}64 \times 10^4$ at 362 nm (λ_{max}) of the synthesized oxazolone

(solvent: the reaction solution) the oxazolone in the assay was formed in 98% yield.

The measurement of the absorbance should be made within 2 h as the oxazolone slowly undergoes alcoholysis, cf. Bennett & Hoerger (1952).

Extraction of acetylsalicylic anhydride from acetylsalicylic acid

De Weck (1971) has described how acetylsalicylic anhydride may be quantitatively extracted from acetylsalicylic acid by dissolving the sample under vigorous stirring in a mixture of equal volumes of benzene and 0·15 M phosphate buffer pH 7·8–8·0, 0·5N sodium hydroxide being continuously added dropwise over a period of about 90 min in order to maintain the pH. We have found that extraction of the anhydride could be more conveniently made with 0·5M phosphate buffer pH 8·0 containing sodium hydroxide in quantities equivalent to the amount of acetylsalicylic acid taken. This procedure makes a dropwise addition of sodium hydroxide during the dissolution period unnecessary and furthermore, it reduces the dissolution period to 1–3 min. The 0·5M phosphate solution pH 11·3 described under reagents contains sodium hydroxide in amounts such that the pH of the solution after dissolution of 0·5 g of acetylsalicylic acid has decreased to 7·8–8·0.

To evaluate the efficiency of the extraction procedure, benzene solutions of acetylsalicylic anhydride was placed in varying amounts in the glass tubes. After evaporation of the benzene under a gentle air current 500 mg of acetylsalicylic acid ("preparation C", see below) was added and the mixture analysed by the general procedure. The percentage recovery of added acetylsalicylic anhydride (10–100 μg) was within the range 98·8–99·8. When benzene solutions of acetylsalicylic anhydride were used as extraction solvents instead of pure benzene for the analysis of the acetylsalicylic acid sample, the percentage recovery of the "added" anhydride was within the range 99·4–100·0.

Precision and sensitivity of the method

To examine the precision of the method, 10 determinations were made on a benzene solution of acetylsalicylic anhydride (without extraction) and on an acetylsalicylic acid sample containing 0·024% of acetylsalicylic anhydride as determined by the method. The relative standard deviations were 1·2% and 1·8%, respectively.

The method is capable of determining the anhydride down to a few micrograms.

Analysis of commercial acetylsalicylic acid samples

Nine different samples of crystalline acetylsalicylic acid marketed in Denmark for clinical use were assayed for their content of acetylsalicylic anhydride by the method. It appears that all the preparations contain acetylsalicylic anhydride impurities, and that preparation A is particularly contaminated compared with the others (Table 1).

To decide whether acetylsalicylic acid might have been transferred in minute amounts to the benzene phase and thereby contributed to the oxazolone formation, attempts were made to obtain an anhydride-free preparation by recrystallization from benzene. However, this procedure proved unsuccessful because after recrystallization of one preparation, sample C, 0·0032% of anhydride was found, which is nearly three times more than that in the untreated sample. De Weck (1971) had noted that heating acetylsalicylic acid in light petroleum led to the formation of acetyl-

Table 1. *Results of analysis for acetylsalicylic anhydride in nine different acetylsalicylic acid preparations commercially available in Denmark.*

Preparation	Acetylsalicylic anhydride (%)
A	0·024 ± 0·0004 (10)
B	0·0016 ± 0·00004 (10)
C	0·0012 ± 0·00002 (4)
D	0·0014 ± 0·00003 (4)
E	0·0029 ± 0·00006 (3)
F	0·0024 ± 0·00005 (3)
G	0·0078 ± 0·0002 (3)
H	0·0066 ± 0·0001 (4)
I	0·0028 ± 0·00006 (3)

Values are means ± standard deviation with the number of determinations in parentheses.

salicylic anhydride and, accordingly, it appears that recrystallization of acetylsalicylic acid from organic solvents may be one way by which the acid becomes contaminated with the anhydride.

On the other hand, an anhydride-free sample of acid could be obtained by using the difference in their susceptibilities towards alkaline hydrolysis. The half-lives for the hydrolysis of the anhydride and acid have been reported to be 9 min at 26° and pH 8·0 (Garrett, 1960) and 55 h at 25° and pH 8 (Garrett, 1957), respectively, and therefore, by dissolving the acid sample (0·5 g) in the phosphate solution, final pH 8·0, and allowing this solution to stand at room temperature for (20–25°) 2·5 h before the 2 ml of benzene are added, the anhydride in the acetylsalicylic acid will be completely hydrolysed while only a very small part of the acetylsalicylic acid is hydrolysed. On assaying preparations A and C in this manner, the measured absorbance for both was zero, thus demonstrating no interference in the method by acetylsalicylic acid.

Another recently discovered impurity in acetylsalicylic acid, acetylsalicylsalicylic acid (Patel, Perrin & Windheuser, 1972) does not interfere in the method, if only for the reason that the compound is fully ionized at pH 8 and therefore is not extracted into the benzene phase.

REFERENCES

BALTAZZI, E. (1955). *Quarterly Review Chemical Society*, **9**, 150–173.

BENNETT, E. L. & HOERGER, E. (1952). *J. Am. chem. Soc.*, **74**, 5975–5979.

CORNFORTH, J. W. (1949). In: *The Chemistry of Penicillin*, pp. 730–848. Editors: Clarke, H. T., Johnson, J. R. & Robinson, R. Princeton, New Jersey: Princeton University Press.

DE WECK, A. L. (1971). *Int. Arch. Allergy*, **41**, 393–418.

GARRETT, E. R. (1957). *J. Am. chem. Soc.*, **79**, 3401–3408.

GARRETT, E. R. (1960). *Ibid.*, **82**, 711–718.

GILLESPIE, H. B. & SNYDER, H. R. (1943). In: *Organic Syntheses, Coll. vol. II*, pp. 489–491. Editor: Blatt, A. H. New York: J. Wiley & Sons.

PATEL, S., PERRIN, J. H. & WINDHEUSER, J. J. (1972). *J. pharm. Sci.*, **61**, 1794–1796.

Pharmacopoea Nordica Edito Danica (1963). Copenhagen: Nyt Nordisk Forlag, Arnold Busck.