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# 1 General Aspects

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## 1

## General Aspects

### 1.1

#### History

##### 1.1.1

#### Willow Bark and Leaves as Antipyretic, Anti-Inflammatory Analgesics

**Medical Effects of Willow Bark** Treatment of diseases by plants or extracts thereof is as old as the history of mankind. This is also true for fever and pain, two particularly frequent and inconvenient symptoms of acute illnesses, as well as arthritis and rheumatism, two examples for chronic painful diseases. Rheumatism already existed in old Egypt, as seen from cartilage alterations in Egyptian mummies. The Egyptians were aware of the pain-relieving effects of potions made from myrtle and willow leaves. Clay tablets from the Sumerian period also described the use of willow leaves for medical purposes. *Hippocrates* recommended leaves of the willow tree for medical purposes about 400 BC. *Pliny* (compilations) and *Dioscurides* (*Materia Medica*) also recommended decocts of willow leaves or ash from the willow bark to treat sciatica (lumbago) and gout in about 100 AD. Outside Europe, it was the Nama (Hottentots) in Southern Africa who had “for a long time” used the bark of willow trees to treat rheumatic diseases (cited after Ref. [1]). This comment was made by Dr Ensor at Cape of Good Hope in his reply to a publication of Dr MacLagan in 1876, describing the use of salicylates for treating rheumatism [2].

The first known communication on the medical use of willow bark extracts in modern times came from Reverend *Edward Stone* from Chipping Norton (Oxfordshire, England). In 1763, he treated some 50 cases of “aigues, fever, and intermitting disorders” with a powdered dry bark preparation of willow tree [3]. The doses were about “20 gr(ains) [1.3 g] to a dram of water every 4 h.” On June 2, 1763, he wrote a letter entitled “An account of the success of the bark of the willow in the cure of aigues” to the Earl of Macclesfield, the then president of the Royal Society of London. In this letter, he summarized his opinion about this treatment as follows:

“... As this tree delights in moist or wet soil where agues chiefly abound, the general maxim, that many natural maladies carry their cure along with them or that their remedies lie not far from their causes, was so very apposite to this particular case, that I could not help applying it; and this might be the intention of providence here, I must own had some little weight with me ...”

After claiming to have obtained good results he concluded:

“... I have no other motives for publishing this valuable specific than that it may have a fair and full trial in all its variety of circumstances and situations, and that the world may reap the benefits accruing from it.”

### 1.1.2

#### Salicylates as the Active Ingredient of Willow Bark and Other Natural Sources

**Plants as Natural Sources of Salicylates** In 1828, German pharmacist *Buchner* was the first to prepare a yellowish mash of bitter taste from boiled willow bark, which he named salicin, after the Latin word for willow (*salix*). He considered salicin as the antipyretic component of willow bark and recommended its use for treatment of fever. A similar conclusion had earlier been reached by the Italians *Brugnatelli* and *Fontana* in 1826 using a less purified preparation of willow bark. They also considered salicin as the active principal component of willow bark (cited after Ref. [4]). In 1830, Frenchman *Leroux* was the first to obtain salicin in crystalline form. Only 3 years later, in 1833, the pharmacist *Merck* in Darmstadt (Germany) announced highly purified salicin from willow bark as an antipyretic for half of the price of quinine (cited after Ref. [5]) – at the time a really attractive offer.

Salicin is not only the antipyretic ingredient of willow bark but also the reason for its bitter taste and irritation of stomach mucosa. Salicin hydrolyzes in aqueous media to glucose and salicylic alcohol (saligenin). Saligenin has no bitter taste and can be easily oxidized to salicylic acid. *Raffaele Piria*, an Italian, was the first to successfully synthesize salicylic acid (acide salicylique ou salicylique) in 1839 from salicin and correctly determined the empirical formula  $C_7H_6O_3$ . This increased the possibility of replacing the poorly palatable salicin with salicylic acid, for example, as a good water-soluble sodium salt. This became practically significant after the new rich natural sources for salicylates were detected. This included wintergreen oil obtained from the American evergreen (*Gaultheria procumbens*) and spireic acid (acidum salicylicum) from the American teaberry (*Spirea ulmaria*). Gaultheria oil (wintergreen oil) contains large amounts of methylsalicylate from which free salicylic acid can easily be obtained.

**Chemical Synthesis of Salicylic Acid** The modern pharmaceutical history of salicylate and its derivatives begins with the synthetic production of the compound. In 1859, *Hermann Kolbe*, a German from Marburg, produced the first fully synthetic salicylic acid from the already known decomposition products phenol and carbonic acid. Kolbe later improved the technology by using sodium phenolate and carbon dioxide under high-pressure conditions at  $140^\circ\text{C}$ ; he was the first to receive a patent for this procedure. Kolbe stimulated his student *Friedrich von Heyden* to further improve the technique to make the compound on an industrial scale. Von Heyden did this in the kitchen of his villa in Dresden (Saxony). The development of a technology to synthesize large amounts of salicylate, independent of the limited availability of natural sources with varying contents and seasonal variations of the active ingredient, opened the door for its broader clinical use and caused a massive drop in price: the price of 100 g of salicylic acid prepared from salicin (gaultheria oil) dropped from 10 to 1 Taler/100 g (Dollar = American for Taler) for the chemical product made through Kolbe's synthesis (cited after Ref. [6]). In 1874, von Heyden founded a factory Salizylsäurefabrik Dr. von Heyden in Radebeul, today part of Dresden (Germany). This factory was extremely successful: after making 4000 kg of salicylic acid in the first year, the annual production was increased to 25 tons only 4 years later. Thus, salicylate became available and known to all civilized countries (cited after Ref. [7]). Interestingly, after solving some legal issues, von Heyden also produced the salicylic acid that was later used by Bayer to make aspirin [8].

**Practical Use of Salicylates** After salicylate as a cheap chemical became available in essentially unlimited amounts, it was tested for several practical applications. For example, salicylic acid was soon found to have antiseptic properties that could be useful to preserve milk and meat. The compound was also recommended as an alternative to carbolic acid, which was the antiseptic of choice in surgery those days. The antipyretic action of

salicylate in infectious diseases was for a time attributed to its antiseptic activity, until it was shown that the sodium salt with little antiseptic properties was an equally effective antipyretic (cited after Ref. [1]). Importantly, salicylic acid was also studied as a potential drug in a large variety of diseases. In 1875, *Ebstein* and *Müller* detected the blood sugar-lowering action of the compound [9]. Shortly thereafter, the uricosuric action of salicylate was described. Thus, salicylates appeared to be useful for treatment of diabetes and gout.

**Salicylic Acid as an Antirheumatic Agent** Of the several discoveries regarding practical applications of salicylates, the most significant was the finding that synthetic salicylates were potent anti-inflammatory analgesics and useful for treating rheumatic diseases. *Franz Stricker* was the first to publish that sodium salicylate was not only an antipyretic remedy but also an effective drug for treatment of rheumatic fever. He introduced salicylate in 1876 as an analgesic antirheumatic drug at the Charité in Berlin [10]. Two months later, Scottish physician *T.J. MacLagan* published the first of a series of articles showing that administration of salicylate to patients with rheumatic fever resulted in the disappearance of pain and fever. Similar results were reported by Frenchman *Germain Sée* 1 year later [11]. These three studies marked the beginning of the widespread therapeutic use of salicylates, here sodium salicylate, as analgesic anti-inflammatory drugs in clinical practice.

### 1.1.3

#### Synthesis of Acetylsalicylic Acid and First Clinical Studies

**The Invention of Acetylsalicylic Acid** Despite the undoubted benefits of sodium salicylate in the treatment of pain, fever, and inflammatory disorders, there were several problems with the practical handling of the compound. These included an unpleasant sweetish taste and, in particular, irritation of the stomach, often associated with nausea and vomiting. Another side effect was hearing

disorders (tinnitus). These side effects were frequent at the high doses of 4–6 g per day, which had to be taken regularly by patients suffering from chronic (rheumatic) pain. Thus, after having an effective technology to generate large amounts of synthetic salicylate, several efforts were made to improve the efficacy of the compound by appropriate chemical modifications, eventually resulting in reduced dosage and improved tolerance. Several groups of researchers addressed this issue with different results [12, 13] (discussed subsequently) until scientists at the Bayer Company in Elberfeld, today in Wuppertal (Germany), succeeded in synthesizing acetylsalicylic acid (ASA) in a chemically pure and stable form. The compound was soon found to be at least as effective as sodium salicylate as an antipyretic analgesic, but was considerably better tolerated.

**The History of Bayer Aspirin** Several individuals at Bayer Company had markedly influenced the development of aspirin. The first was *Arthur Eichengrün* (Figure 1.1). He joined the Bayer Company in 1895 and became head of the newly founded Pharmaceutical Research Department [14]. According to a report published 50 years later [15], it was he who had the idea to acetylate salicylate to improve its efficacy and tolerance. This concept of acetylation of drugs to improve their efficacy was not new at the time and had already been successfully used to synthesize phenacetin from *p*-aminophenol, a considerably more powerful analgesic.



Arthur Eichengrün (1867–1949)



Felix Hoffmann (1868–1946)

**Figure 1.1** Arthur Eichengrün and Felix Hoffmann.

*Felix Hoffmann* (Figure 1.1) was the chemist working on this issue in Eichengrün's group, and it was he who originally worked out a new technology of the acetylation reaction of salicylate (and other natural products such as guaiacole, cinchonine, and morphine). He was the first to produce chemically pure and stable ASA on August 10, 1897, according to his handwritten notes in the laboratory diary (Figure 1.2).

Two other individuals have to be mentioned in this context: *Heinrich Dreser*, head of the Department of Pharmacology at Bayer, and *Carl Duisberg*, the then head of the research and later president of the Bayer Company. Dreser was not interested in this kind of research. Initially, he was also not informed by the pharmacists about the successful clinical testing of the new compound, although according to his contract with Bayer, the pharmacists should have had reported this finding to him. He was probably not amused to hear about these results. According to Eichengrün [15] and other sources, he did everything to block the further development of the compound. Duisberg, the key manager in charge, emphatically supported the activities of Eichengrün and Hoffmann. The further development and clinical introduction of aspirin as an antipyretic analgesic, eventually resulting in the worldwide spread of the compound, is his merit. The new drug received the trade name "aspirin," which is composed from "acetic" and "spireic acid," a former name of *o*-hydroxybenzoic acid (salicylic acid), originally prepared from *S. ulmaria*, the richest natural source of salicylates.

The first description of the pharmacology of aspirin was published in 1899 by Dreser [16]. The names of the inventors Hoffmann and Eichengrün were not mentioned in this paper. Dreser considered aspirin as a better tolerable prodrug of the active principal salicylic acid. This was basically correct even from today's viewpoint for the symptoms the substance was supposed to be used at the time. According to Eichengrün [15], Dreser had nothing to do with the invention. Interestingly, it was neither Eichengrün nor Hoffmann but Dreser

who had the financial benefits from the discovery. According to a contract with Bayer, the products invented under the direction of Eichengrün had to be patented in Germany to get a royalty for the inventor from the company [15]. Acetylsalicylic acid was registered on February 1, 1899 under the trade name "Aspirin" by the Imperial Patent Bureau (Kaiserliches Patentamt) in Berlin and a few weeks later introduced as a tablet (1 tablet = 5 gr(ain) 325 mg) in Germany. However, the compound did not receive recognition as a substance to be patented in Germany. Aspirin was patented in 1900 exclusively in the United States (Figure 1.3). This patent expired in 1917. In 1918, after the World War I ended, Bayer's assets were considered enemy property, confiscated by the US government, and auctioned in the United States the same year [17]. It took Bayer about 80 years to buy back the rights of the trade name "Aspirin."

A recent article raised doubts about Hoffmann as the inventor of aspirin and ascribed this merit to Eichengrün [18]. Eichengrün was Jewish and lost the fruits of his scientific research, including the invention of several other products in addition to aspirin, such as acetate silk, because of political reasons during the Nazi regime after 1933. Eichengrün was interned in 1944 in a concentration camp and remained there until the end of World War II. In 1949, the year of his death, Eichengrün stated in an article, published in the German scientific journal *Die Pharmazie*, that it was he and Felix Hoffmann who should be considered as the inventors of aspirin. Though Hoffmann was probably the person who designed and did the experiments eventually resulting in pure and chemically stable ASA – additionally evidenced by the (obviously undisputed) sole mention of his name on the patent application in 1900 (Figure 1.3) – one should also ascribe a significant contribution to Eichengrün.

**Further Attempts to Make Acetylsalicylic Acid** At this point, it should be noted that Hoffmann was not the first person who tried to chemically synthesize ASA. In 1853, Frenchman *Charles Frédéric Gerhardt* from Straßburg (Alsace) described the synthesis of a new compound from acetyl chloride and sodium salicylate, which he named Salicylate acétique (see Ref. [19]).



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Aspirin - Bericht

Dr. Hoffmann

Acetylsalicylsäure.

Kauf man 100,0 Salicylsäure mit 150,0 Acetanhydrid 3  
 Stunden unter Rückfluss, so ist die S. quantitativ  
 ausfällbar. Auf Versuchen die Essigsäure selbst in dieselbe  
 in Wasser, die aus 646 kryoskopisch 136° schmelzen  
 (Literaturangabe ist 118°) Zu Beginn der ersten Uebersch  
 in Schmelze gibt das reine Acetylproduct keine Flüssig-  
 keit nach dem Kochen, weshalb sie sich leicht in der Salicyl-  
 säure unterkühlt. Auf die physikalischen Eigenschaften  
 wie zum Beispiel Gefrierpunkt oder die Löslichkeit unterkühlt  
 sich das Acetylsalicylsäure vollständig nur der Salicylsäure  
 und nicht dieselbe nachher. Dieser auf die Temperatur bei gezeig.

Elberfeld, den 10. Okt. 1897

F Hoffmann

F H

This publication of Gerhardt was used by several authors to ascribe the invention of ASA to him (e.g., [1, 20]). This appears not to be correct for several reasons. His preparation of ASA was impure, due to the insufficient technical procedure used by him [12], and rather a labile, intermediate raw product of the reaction between acetyl chloride (prepared by him by a suboptimal procedure) and sodium salicylate. The chemical structure was not determined. Because of inappropriate processing of the raw product, Gerhardt only obtained salicylic acid as a stable end product. He concluded that acetylated salicylic acid is unstable and in water immediately breaks down to salicylic acid and acetate. Both statements are wrong and do not qualify Gerhardt for the claim to have invented the synthesis of ASA [7].

In 1859, *H. von Gilm*, a pharmacist from Innsbruck (Austria), reported the synthesis of ASA as did *Karl Kraut* 10 years later in 1869. Again, these preparations were impure – see also comments in the patent application of Hoffmann (Figure 1.3). During the following 20 years, there were apparently no further attempts to improve the synthetic procedure to obtain ASA as a pure, chemically stable compound.

Thus, the origin of ASA, in contrast to the natural product salicylic acid, was exclusively in organic chemistry. From the point of view of an organic chemist, the substance had no obvious practical benefit, and there were definitely no ideas or even concepts about its possible use as a therapeutic agent. Thus, ASA probably would have suffered the fate of several hundreds of chemicals before and many thousands thereafter – a product of chemical synthesis, principally easy to make but more difficult to generate in pure and chemically stable form and without any practical significance. On the contrary, Hoffmann and Eichengrün, combined their knowledge about the chemistry of a natural product with synthetic chemistry with the intention to make a new drug out of it with improved pharmacological properties. These studies would probably not have been done without the support of the Bayer Company. Therefore, the company had good reason to celebrate the 100th anniversary of the compound, which in the meantime became the most popular drug in the world [18].

In this context, an interesting comparison with the discovery of prostacyclin can be made. Its chemical structure and a suggested (later confirmed) enzymatic synthetic pathway were originally described in 1971 by Pace-Asciak and Wolfe. These authors considered this (labile) product as just another prostaglandin, in addition to dozens of already known compounds, which was possibly overlooked by earlier investigators because of its low biological activity. This was tested at that time in bioassay experiments using the rat stomach strip. It also remained uncertain whether the compound was synthesized at all in the intact stomach wall and, if so, was released in biologically active amounts [21].

A completely different approach was followed by the group around Sir John Vane. The group's work on prostacyclin started with the discovery of a biological effect – inhibition of platelet aggregation – of an enzymatic product made from prostaglandin endoperoxides on artery walls. This prostaglandin, originally named PGX, differed in its biological behavior from all other known prostaglandins [22]. PGX was later identified as the already known enzymatic product of prostaglandin endoperoxides, described by Pace-Asciak and Wolfe, and was renamed as prostacyclin (PGI<sub>2</sub>). Despite the originality and merits of Pace-Asciak and Wolfe regarding the detection of biosynthetic pathways of natural prostacyclin and its chemical structure, the medical history of prostacyclin starts with the work of Vane's group, which was the first to discover the biological significance of prostacyclin for control of hemostasis.

### The Introduction of Acetylsalicylic Acid into the Clinics

*Kurt Witthauer*, a specialist in internal medicine in a city hospital (Diakonie Krankenhaus – still existent!) in Halle/Saale (Germany), and *Julius Wolgemuth* [23] from Berlin published the first clinical investigations on aspirin in 1899. Witthauer began his report as follows:

“... Nowadays, certain courage is necessary to recommend a new drug. Almost every day those are thrown on the market and one has to have an excellent memory to keep all the new names and brands in mind. Many drugs appear, are praised and recommended by companies and certain authors but after a short time have disappeared without any further comments [24].”

The author also did not forget to instruct his readers that he did this study with “not little distrust.”

# UNITED STATES PATENT OFFICE.

FELIX HOFFMANN OF ELBERFELD GERMANY ASSIGNOR TO THE FARBEN-FABRIKEN OF ELBERFELD COMPANY OF NEW YORK.

## ACETYL SALICYLIC ACID.

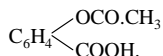
SPECIFICATION forming part of Letters Patent No. 644,077, dated February 27, 1900.

Application filed August 1, 1898 Serial No. 087,385 (Specimens.)

*To all whom it may concern:*

Be it known that I, FELIX HOFFMANN, doctor of philosophy, chemist, (assignor to the FARBENFABRIKEN OF ELBERFELD COMPANY, of New York) residing at Elberfeld, Germany, have invented a new and useful Improvement in the Manufacture or Production of Acetyl Salicylic Acid; and I hereby declare the following to be a clear and exact description of my invention.

In the *Annalen der Chemie und Pharmacie*, Vol. 150, pages 11 and 12, Kraut has described that he obtained by the action of acetyl chlorid on salicylic acid a body which he thought to be acetyl salicylic acid. I have now found that on heating salicylic acid with acetic anhydride a body is obtained the properties of which are perfectly different from those of the body described by Kraut. According to my researches the body obtained by means of my new process is undoubtedly the real acetyl salicylic acid

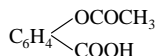


Therefore the compound described by Kraut cannot be the real acetyl salicylic acid but is another compound. In the following I point out specifically the principal differences between my new compound and the body described by Kraut.

If the Kraut product is boiled even for a long while with water, (according to Kraut's statement,) acetic acid is not produced, while my new body when boiled with water is readily split up, acetic and salicylic acid being produced. The watery solution of the Kraut body shows the same behavior on the addition of a small quantity of ferric chlorid as a watery solution of salicylic acid when mixed with a small quantity of ferric chlorid—that is to say, it assumes a violet color. On the contrary, a watery solution of my new body when mixed with ferric chlorid does not assume a violet color. If a melted test portion of the Kraut body is allowed to cool it begins to solidify (according to Kraut's statement) at from 118° to 118.5° centigrade while a melted test portion of my product solidifies at about 70° centigrade. The melting-points of the two compounds cannot be compared be-

cause Kraut does not give the melting-point of his compound. It follows from those details that the two compounds are absolutely different.

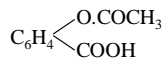
In producing my new compound I can proceed as follows, (without limiting myself to the particulars given:) A mixture prepared from fifty parts of salicylic acid and seventy-five parts of acetic anhydride is heated for about two hours at about 150° centigrade in a vessel provided with a reflex condenser. Thus a clear liquid is obtained, from which on cooling a crystalline mass is separated, which is the acetyl salicylic acid. It is freed from the acetic anhydride by pressing and then recrystallized from dry chloroform. The acid is thus obtained in the shape of glittering white needles melting at about 135° centigrade, which are easily soluble in benzene, alcohol, glacial acetic acid, and chloroform, but difficultly soluble in cold water. It has the formula



and exhibits therapeutical properties.

Having now described my invention and in what manner the same is to be performed, what I claim as new, and desire to secure by Letters Patent, is—

As a new article of manufacture the acetyl salicylic acid having the formula:



being when crystallized from dry chloroform in the shape of white glittering needles, easily soluble in benzene, alcohol and glacial acetic acid, difficultly soluble in cold water being split by hot water into acetic acid and salicylic acid, melting at about 135° centigrade substantially as herein before described.

In testimony whereof I have signed my name in the presence of two subscribing witnesses.

Witnesses:  
R. E. JAHN,  
OTTO KÖNIG.

FELIX HOFFMANN.

Figure 1.3 The acetyl salicylic acid patent.

However, his impressions about the results were obviously quite positive and he came to the conclusion:

“... According to my positive results, the company is now prepared – after waiting for a long while – to introduce the new compound on the market. I sincerely hope that the difficult technology to make it will not cause a too high price, to allow the broad general use of this valuable new drug.”

**Aspirin as a Household Remedy Against Fever, Inflammation, and Pain** Soon after the introduction of ASA into medical use under the brand name “aspirin,” the new drug became a very popular remedy against fever, inflammation, and pain. A local German newspaper, *Kölner Stadtanzeiger*, published the following recommendation for treatment of flu on March 6, 1924:

“... As soon as you feel yourself ill, you should go to bed and have a hot-water bottle at your feet. You should drink hot chamomilae tea or grog in order to sweat and should take 3 tablets of aspirin a day. If you follow these instructions you will recover within a few days, in most cases ...”

This extract is remarkable for several reasons: during the past 25 years of practical use, aspirin had become a drug whose name was not only well known to health professionals but also to the general public. Certainly, the flu pandemic with millions of victims alone in Europe at the beginning of the last century as well as the limited availability of antipyretic analgesics other than aspirin contributed to this. However, the compound was generally recommended – and accepted – by the lay man and doctors – as a “household remedy” for treating pain, fever, inflammation, and many other kinds of “feeling bad,” although essentially nothing was known about the mechanisms of action behind these multiple activities of the drug. It was only in the 1950s, when the first report was published, that salicylates including aspirin at anti-inflammatory doses uncouple oxidative phosphorylation in a number of organs and tissues [25]. However, this at the time was con-

sidered to be mainly of toxicological interest. Whether this contributes to the clinical efficacy of aspirin as anti-inflammatory agent is still unknown.

#### 1.1.4 Mode of Aspirin Action

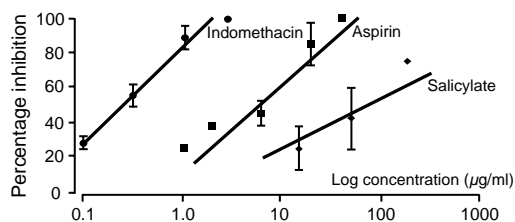
**Aspirin and Prostaglandins** In 1971, the journal *Nature* published three articles of the group of *John Vane* at the Royal College of Surgeons of England. These articles demonstrated for the first time a mechanism of action of aspirin that explained the multiple biological activities of the compound by one single pharmacological effect: inhibition of prostaglandin biosynthesis [26]. In his pioneering paper, the later Sir John Vane showed by elegant bioassay experiments that aspirin – and salicylate – inhibited prostaglandin formation in cell-free systems after tissue injury (Figure 1.4). This finding and his later discovery of prostacyclin were honored with the Nobel Prize for Medicine in 1982.

#### Inhibition of Prostaglandin Synthesis as a Mechanism of Action for Aspirin-like Drugs

J. R. VANE

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**Figure 1.4** First description of inhibition of prostaglandin biosynthesis by aspirin and salicylate and the reference compound indomethacin by John Vane. Note the dose dependency of this reaction by all compounds including aspirin (modified after [26]).

Prostaglandins, thromboxane A<sub>2</sub>, and leukotrienes are members of a group of natural lipid mediators that are generated by oxidation from arachidonic acid. Because of this origin, they all have a 20-carbon backbone and

are summarized as “eicosanoids” (Greek: *eikos* = twenty). Today, more than 150 eicosanoids are known and have been structurally identified. Arachidonic acid, the precursor fatty acid, is a constituent of the cell membrane phospholipids and is released from them by phospholipases. Eicosanoid synthesis starts with the availability of free arachidonic acid.

The first oxidation step of arachidonic acid to generate prostaglandins is catalyzed by cyclooxygenases (COXs). These enzymes are widely distributed throughout the body. The primary products – the prostaglandin endoperoxides – are then converted to the terminal products of this pathway, that is, prostaglandins and thromboxane A<sub>2</sub>, in a more cell-specific manner. The active products are not stored but released, act on their cellular target, and are afterward degraded enzymatically.

Prostaglandins exert their multiple actions via specific G-protein-coupled receptors at the cell surface. The direction and intensity of these actions is determined by the kind and the number of available prostaglandin receptors from which today about 10 are known. Prostaglandins act as local mediators that dispatch signals between cells. Thus, prostaglandin generating cells, and these are probably all cells of the body, do not require prostaglandin biosynthesis for survival. Consequently, prostaglandins are not essential for vital functions, such as energy metabolism or maintenance of the cell cytoskeleton.

The cellular prostaglandin synthesis can be markedly increased in response to disturbed homeostasis (injury) to adapt cellular functions to changes in the environmental conditions. An increased prostaglandin synthesis “on demand,” therefore, reflects a tissue-specific response to increased needs. Examples for physiological stimuli are hemostasis and pregnancy, whereas the increased prostaglandin production in inflammation, atherosclerosis, and tumorigenesis rather reflects the response to pathological stimuli.

Thus, any change in generation of prostaglandins or the related thromboxanes *per se* is neither good nor bad but rather reflects a functioning cell-based adaptation or defense mechanism. Functional disorders may arise, when prostaglandins become limiting factors for control of cell and organ function, respectively. Thus, any pharmacological interference with these processes may be either positive or negative but in most cases is not associated with any measurable functional change at the organ level as long as other mediator systems can compensate for it.

**Aspirin and Cyclooxygenases** Aspirin blocks the biosynthesis of prostaglandins and thromboxane A<sub>2</sub> at the level of prostaglandin endoperoxides or

cyclooxygenase(s) by irreversible acetylation of a critical serine in the substrate channel of the COX enzyme (Section 2.2.1). This limits the access of substrate (arachidonic acid) to the catalytic active site of the enzyme [27] and explains the antiplatelet action of the substance, first described by the group of *Philip Majerus* [28]. The group of *William Smith* and *David DeWitt* detected this unique mechanism of action and has made major other contributions to this issue. The contributions of William Smith in elucidating the molecular reaction kinetics of aspirin were acknowledged with the Aspirin Senior Award in 1997.

Two genes have been identified that encode for cyclooxygenases: COX-1 and COX-2. In addition, there is a steadily increasing number of splice variants of these two genes. They are also transcriptionally regulated and might cause synthesis of gene products. Both COX isoforms are molecular targets for aspirin. However, the inhibition of COX-1 appears to dominate at lower concentrations of the compound, whereas aspirin and its primary metabolite salicylate are about equipotent inhibitors of COX-2 (Section 2.2.1). Thus, aspirin contains two pharmacologically relevant groups: the reactive acetyl moiety and salicylate. Both components are biologically active and act independently of each other at different sites. The molecular interaction of aspirin with COX-1 was further elucidated after the crystal structure of the enzyme became clarified by *Michael Garavito* and his group. The contribution of *Patrick Loll* to this work was acknowledged with the Aspirin Junior Award [29].

The detection of inhibition of prostaglandin synthesis was the first plausible explanation for the multiple pharmacological actions of aspirin via an ubiquitous class of endogenous mediators, prostaglandins and thromboxanes. With increasing knowledge of the complex nature of these reactions, specifically the multiple interactions of prostaglandins with other mediator systems, some details of these findings are now interpreted in a different way. This is particularly valid for

the anti-inflammatory activities of aspirin, which are mainly due to the formation of the more stable metabolite salicylate [30] and, possibly, the generation of aspirin-triggered lipoxin (ATL), resulting from the interaction of aspirin-treated (acetylated) COX-2 and the 5-lipoxygenase from white cells (Sections 2.2.1 and 2.3.2). This eventually resulted in the detection of resolvins, a new class of anti-inflammatory mediators, also involved in aspirin action by *Charles Serhan* and his group. The contributions of *Jose Claria* to this work [31] were acknowledged with the Aspirin Junior Award in 1996.

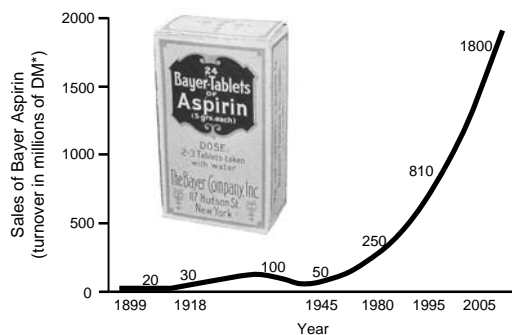
**Aspirin and Gene Transcription** After the discovery of the inducible isoform of COX-2, it became rapidly clear that aspirin was rather ineffective on this enzyme at low antiplatelet concentrations, and higher doses were required to suppress COX-2-dependent prostaglandin formation. This finding also confirmed the clinical experience that the analgesic and anti-inflammatory effects of the compound require substantially higher doses than those necessary for inhibition of platelet function. *Kenneth Wu* and colleagues were the first to show that aspirin and salicylate interact with the binding of transcription factors to the promoter region of the COX-2 gene [32]. These factors regulate the gene expression level after stimulation by inflammatory mediators. Later work of *Xiao-Ming Xu* and others of this group eventually identified the binding of CCAAT/enhancer-binding protein- $\beta$  (C/EBP- $\beta$  or NF/IL-6) as one critical control mechanism [33, 34]. It becomes now increasingly evident that the anti-inflammatory and antineoplastic actions of salicylates and aspirin, respectively, involve inhibition of COX-2 gene transcription because COX-2 overexpression is an important permissive factor in these disorders and also might generate compounds other than prostaglandins (Section 4.3.1). The molecular mechanisms of control of inducible COX activity are still under intense research. However, it is interesting to note that the upregulation of salicylate biosynthesis by plants – the natural sources of salicylates – represents a

most effective, transcriptionally regulated defense system that becomes activated in response to about all kinds of noxious stimuli and exhibits a number of similarities to the prostaglandin pathway in animals and men (Section 2.2.2).

### 1.1.5

#### Anti-Inflammatory/Analgesic Actions of Aspirin

The disclosure of a causal relationship between inhibition of prostaglandin synthesis and anti-inflammatory/analgesic actions of aspirin was not only a satisfactory explanation for its mode of action but also a stimulus for the mechanism-based drug research. These new compounds should be able to block prostaglandin biosynthesis via COX inhibition and should also be more potent than aspirin to allow lower dosing at increased efficacy and reduced side effects. Indomethacin was the first of these so-called aspirin-like drugs [35] and was already used as a reference compound in the pioneering experiments of *John Vane* (Figure 1.4). Many others followed too. In 2005, there were more than 20 chemically defined substances on the German market, designed and developed as inhibitors of prostaglandin biosynthesis and approved for clinical use as antipyretic/anti-inflammatory analgesics. There were more than 40 (!) brands containing ibuprofen, 35 containing diclofenac, and 12 containing indomethacin, most of them available in several different galenic preparations. However, there were less than 20 preparations containing aspirin as the only active ingredient. Thus, the invention of aspirin did significantly stimulate basic research for new anti-inflammatory analgesics and still does so as is evident from the detection of ATLs and resolvins. Nevertheless, the use of aspirin has also remarkably increased after the discovery of its prostaglandin-related mode of action (Figure 1.5). Today, ASA in its different commercial preparations is still among the most frequently used antipyretic analgesics for self-medication of headache, flu, and other acute inflammatory/painful states. According to a recent survey in Germany (MONICA registry), salicylates



**Figure 1.5** From 1899 to 2005 (projected) turnover of Bayer aspirin in millions of DM (Deutsche Mark). (\*): 1 DM (Deutsche Mark) is equivalent to 0.51 € or 0.67 USD (January 2007) [37].

still keep the key position (70–80%) in OTC drugs for these symptoms [36].

### 1.1.6

#### Aspirin in the Cardiovascular System

**Bleeding Time and Platelet Function** Aspirin was in clinical use for about half a century, when the first reports about disturbed hemostasis were published. In 1945, *Singer*, an ETN specialist, reported late bleedings after tonsillectomies [38]. He attributed this to his prescription of aspirin for analgesic purposes. Withdrawal of aspirin or its replacement by metamizol (dipyrone) resulted in disappearance of bleeding. A relationship between aspirin intake and bleeding was also considered for tooth extractions and epistaxis [39, 40]. *Beaumont* and *Willie* [41] reported that aspirin prolonged bleeding time in patients with cardiac diseases. *Quick* and *Clesceri* [42] suggested that this effect of high-dose (6 g) aspirin might be caused by the reduction of a stable procoagulatory factor in plasma, probably prothrombin. In 1967, *Quick* demonstrated that a prolonged bleeding time was specific for aspirin and was not seen after salicylate was administered [43]. Similar results were obtained by the group of *Mustard* [44]. These authors confirmed the results of *Quick* and extended them also to several animal

species. In addition, they showed that the antiplatelet effect of aspirin depends on the kind of platelet stimulus. Specifically, aspirin did not inhibit ADP-induced primary platelet aggregation, a finding that was largely ignored later in many platelet function assays *in vitro* (Section 2.3.1). During 1967–1968, *Weiss* and colleagues, *Zucker* and *Peterson*, and *O'Brien* published the first more systematic studies on the action of aspirin on platelet function in healthy men [45–48]. *O'Brien* found a significant inhibition of platelet function at the “subclinical” dose of 150 mg and strongly recommended a clinical trial on the compound in patients at elevated thrombotic risk.

#### Mechanisms of Antiplatelet Action of Aspirin

The elucidation of the mechanism of action of aspirin as an antiplatelet drug starts with a study by *Bryan Smith* and *Al Willis* [49], both working at the time in the laboratory of *John Vane*. These authors were the first to show that inhibition of platelet function by aspirin was associated with the inhibition of prostaglandin biosynthesis and concluded that this explains the antiplatelet effects of the compound. At this time, thromboxane was still unknown. A few years later, *Roth* and *Majerus* [28] showed that aspirin causes irreversible acetylation and inhibition of platelet cyclooxygenase. The group of *Garret FitzGerald* [50] confirmed this finding as well as serine<sub>529</sub> (530) in the COX substrate channel as acetylation site for the cloned enzyme from human platelets. These studies provided the rationale for the use of aspirin as an antiplatelet drug in the secondary and primary prevention of atherothrombotic vessel occlusion, specifically myocardial infarction and ischemic stroke (Sections 4.1.1 and 4.1.2). Preeclampsia is another indication for prophylactic aspirin administration after the description of a positive effect of aspirin in high-risk patients by *Crandon* and *Isherwood* [51] and its confirmation in a randomized trial by *Beaufils* and colleagues 6 years later [52] (Section 4.1.5).

**Aspirin and Prevention of Myocardial Infarction and Stroke** In 1949, *Paul C. Gibson* reported for the first

time the successful use of aspirin for prevention of anginal pain and coronary thrombosis [53]. This report was based on a questionnaire sent by him to 20 doctors: fifteen of them had already successfully used the compound for this condition and all of them considered aspirin as “valuable” or “very valuable,” specifically with respect to its analgesic properties. Gibson explained these beneficial effects by a combination of anticoagulatory and analgesic properties of the compound. The recommended doses were 1300 mg (20 grain) or 650 mg (10 grain) aspirin.

The first larger and more systematic investigation of the significance of antithrombotic effects of aspirin for the prevention of myocardial infarctions was published in 1950 by *Lawrence Craven* [54] (Figure 1.6), a suburban general practitioner from Glendale (California) [55].



**Figure 1.6** Photograph of Dr Lawrence L. Craven in 1914, at the age of 31, when he graduated from the University of Minnesota College of Medicine and Surgery (with permission of the University of Minnesota).

His finding reads in the original contribution as follows:

“... during the past two years, I have advised all of my male patients between the ages of 40 and 65 to take from 10–30 grains [650–1950 mg] of acetylsalicylic acid daily as a possible preventive of coronary thrombosis. More than 400 have done so, and of these none has suffered a coronary thrombosis. From past experience, I should have expected at least a few thrombotic episodes among this group. There would appear to be enough evidence of the antithrombotic action of acetylsalicylic acid to warrant further study under more carefully controlled conditions ...”

In the following years, Craven increased the number of his patients to about 8000 – still without having seen any myocardial infarction – and recommended the agent also for prevention of stroke [56, 57]. Unfortunately, he died in 1957, 1 year after publication of his last study, at the age of 74 from a heart attack – despite regularly using aspirin [58].

Craven’s study was a stroke of luck in several aspects: first, he treated exclusively males at an age of increased risk for myocardial infarction who, according to the current knowledge, benefit most from aspirin prophylaxis. He used a dose of aspirin that was high, but in comparison to anti-inflammatory doses at the time for treatment of chronic inflammatory diseases, was rather low. Thus, not too many side effects were to be expected, which was good for the compliance of his patients. Finally, he had no problems with statistics because there were no infarctions in the patient group.

Unfortunately, these data did not find the necessary attention during the following 20 years – possibly due to the low impact factor of the journals where they were published and the fact that Craven himself died of heart attack despite regularly taking aspirin. Until the 1970s of the last century, the significance of thrombosis against spasm for the genesis of myocardial infarction was also in question. Until 1988, more than 15 000 patients were studied in seven placebo-



controlled trials for the secondary prevention of myocardial infarction at the cost of many millions of dollars. None of these studies was significant on its own, possibly because, from today's viewpoint, of poor study design, the highly variable aspirin doses (300–1500 mg/day), the apparently absent systematic control of patient compliance, and a highly variable time point when aspirin treatment was started, in one study (AMIS) up to 5 years (!) after the acute event [59].

These data finished the discussion on the possible use of aspirin for the prevention of myocardial infarctions. In addition, infrequent though severe side effects such as GI or cerebral bleeding and a suggested though never established relationship with Reye's syndrome (Section 3.3.3) have tainted its reputation and resulted in its removal from the list of essential drugs by the WHO in 1988. Ironically, at about the same time, that is, 1988/1989, the first prospective randomized placebo-controlled trial – the US American Physicians' Health Study (Section 4.1.1) – and the subsequent meta-analyses by the Antiplatelet Trialists' Collaboration were published and showed a significant reduction of the incidence of myocardial infarction or other atherothrombotic events in both healthy volunteers and patients at elevated cardiovascular risk [60]. The ISIS-2 study of 1989 convincingly demonstrated for the first time a significant reduction in infarct mortality by aspirin and resulted in the first official guideline recommendation of aspirin use in these patients [61]. The prevention of atherothrombotic vessel occlusions by aspirin in patients at increased vascular risk is now a therapeutic standard. The medical decision to use aspirin in the individual patient is determined by the individual benefit/risk ratio. This issue is particularly relevant in the primary prevention in patients with a low risk profile (Section 4.1.1).

### 1.1.7

#### Current Research Topics

**Clinical Applications** In 1988, *Gabriel Kune* from Melbourne (Australia) published the first report on

reduced incidence of colorectal carcinoma by about 40% in regular (daily) aspirin users as compared to those who did not regularly take the drug [62]. These data were generated in a retrospective, exploratory case–control study, which also noted a significant risk reduction in patients using nonsteroidal anti-inflammatory drugs (NSAIDs) other than aspirin. These data were later confirmed in a large epidemiological trial [63]. However, there are still not sufficient prospective randomized trials to calculate the individual benefit/risk ratio, considering the long treatment period of at least 10 years before significant improvements can be expected. Furthermore, all of the three available randomized trials have determined the (re)occurrence of colorectal adenomas as surrogate parameters for colorectal malignancies (Section 4.3.1). Another mutually interesting therapeutic option is Alzheimer's disease [64] (Section 4.3.2).

**Basic Research** One of the issues of interest in basic research with a considerable clinical concern is the so-called aspirin resistance, that is, a reduced antiplatelet activity of aspirin at antithrombotic doses. Any reduced pharmacological activity will also limit its clinical efficacy in thrombosis prophylaxis. Recent evidence, however, suggests that the clinical significance of this phenomenon might be much less than originally anticipated and, in contrast to “resistance” to clopidogrel, will not require systematic screening (Section 4.1.6).

A particular pharmacological property of aspirin that is not shared with either natural salicylate or coxibs is the acetylation of COX-2 and the subsequent generation of “aspirin-triggered lipoxin” ATL, an anti-inflammatory mediator. This may help better understand and explain clinically well-known phenomena, such as adaptation of stomach mucosa to long-term (high-dose) aspirin use (Section 3.2.1) [65] and the inhibition of neutrophil recruitment to an inflamed site [31] (Section 2.3.2). These activities might be relevant to all clinical situations with an upregulated COX-2, including acute and chronic inflammation, tumorigenesis, and advanced atherosclerosis.

Finally, the newly discovered actions of aspirin on gene regulation are of considerable interest. Control of gene expression by preventing binding of transcription factors to selected areas in the promoter region of genes is not limited to COX-2, but may also occur with other genes that are regulated by the same transcription factors, such as inducible NO synthase or other “immediate early genes” that are involved in rapid adaptation of cell function to changes in the environment. Targeted

control of gene expression and function appears to be pharmacologically much more attractive – and efficient – than just the inhibition of activity of selected enzymes. Salicylates in plants are transcriptionally regulated “resistance genes” and form an essential part of cellular defense mechanisms. Research on these pleiotropic actions of aspirin in man is also a pharmacological challenge [66] and, eventually, might result in the design and development of new class(es) of “aspirin-like” drugs.

### Summary

Extracts or other preparations from willow bark or leaves have been used since ancient times for the treatment of fever, inflammation, and pain. These ancient uses have been rediscovered in modern times. The identification of salicylates as the active fraction eventually resulted in its chemical synthesis, allowing broad-spectrum practical use.

The availability of synthetic salicylate was also the precondition for chemical modification of its structure to increase the activity and to reduce side effects. In this respect, the first successful synthesis of chemically pure and stable ASA by Felix Hoffmann, working in the group of Arthur Eichengrün at Bayer in 1897, was the key event. The new compound was introduced into the market in 1899 under the trade name “Aspirin” and soon became a well-known and widely accepted household remedy for the treatment of pain, fever, inflammation, and almost every kind of “feeling bad.”

The first pharmacological explanation for these multiple actions was provided by the discovery of Sir John Vane in 1971 that aspirin blocked prostaglandin biosynthesis. This explained the analgesic/anti-inflammatory properties of aspirin and other salicylates, which according to the current knowledge, are probably due to the inhibition of COX-2. Interestingly, acetylation of salicylates added a

new property to the compound, which is not shared by any natural salicylate – transacetylation of target proteins, most notably COX-1 in platelets – with subsequent inhibition of platelet function. Inhibition of platelet function is the rationale for the widespread use of aspirin in the prevention of thromboembolic events. Acetylation of COX-2 might result in the generation of ATL, an inhibitor of leukocyte recruitment that facilitates resolution of inflammation.

Current areas of interest in basic and clinical research on aspirin include “aspirin resistance,” its definition, measurement, and significance and the possible clinical benefit of aspirin in the prevention of malignant disorders such as colorectal carcinomas and Alzheimer’s disease. The mode of action of aspirin appears here to be more complex and involves, in addition to inhibition of enzyme activity, actions on gene regulation. Targeted modulation of cytokine- and tumor promoter-induced upregulation of “early response” genes, such as COX-2 or iNOS and probably others, by aspirin and salicylates appears to be much more attractive and promising than just inhibition of enzyme activity. In this way, salicylate acts in plants as a transcriptionally regulated “resistance gene.” Transfer of this principle to the animal kingdom and men, eventually, might result in the design and development of new and even more effective class(es) of “aspirin-like” drugs.

**Table 1.1** The history of salicylates and acetylsalicylic acid.

Date	Event
400 BC–100 AD	<i>Hippocrates</i> recommends bark and leaves of the willow tree ( <i>Salix alba</i> ) for medical use. This recommendation is later encyclopedized by <i>Pliny</i> and <i>Dioscurides</i> as popular medical knowledge of the time.
1763	Rev. <i>Edward Stone</i> recommends the use of willow bark extracts for treatment of “Aigues and intermitting disorders.”
1826–1830	<i>Brugnatelli</i> and <i>Fontana</i> as well as <i>Buchner</i> identify salicin as the active antipyretic ingredient of the willow bark. <i>Leroux</i> in 1830 is the first to isolate salicin in crystalline form. The compound, prepared from willow barks, is later sold by Ernst <i>Merck</i> as an antipyretic drug for half the price of quinine.
1839	<i>Piria</i> prepares salicylic acid from salicin and correctly determines the brutto formula $C_7H_6O_3$ .
1835–1843	New rich sources of natural salicylates are detected, most notably wintergreen oil from the American evergreen ( <i>G. procumbens</i> ), containing about 99% methylsalicylate. This finding markedly improves the availability of salicylates for practical use.
1859–1874	<i>Kolbe</i> synthesizes for the first time pure salicylate from the already known decomposition products phenol and carbonic acid. His student <i>von Heyden</i> improves the technology of synthesis and founds the first salicylic acid producing factory in Radebeul (Dresden) in 1874. The plant rapidly produces tons of salicylic acid. This provides unlimited amounts of the compound independent of natural sources for medical use.
1875	<i>Ebstein</i> and <i>Müller</i> detect the blood sugar-lowering action of salicylates.
1876	<i>Stricker</i> introduces salicylate as an analgesic/antirheumatic drug at the Charité in Berlin. Shortly thereafter, Scottish physician <i>MacLagan</i> and Frenchman <i>Germain Sée</i> from Strassburg (Alsace) also describe an antipyretic/analgesic activity of the compound.
1897	<i>Felix Hoffmann</i> , working in the pharmaceutical research group at Bayer laboratories in Elberfeld under direction of <i>Arthur Eichengrün</i> , synthesizes for the first time acetylsalicylic acid as a chemically pure and stable compound.
1899	<i>Heinrich Dreser</i> , head of the pharmacological research laboratories at Bayer, publishes the first report on the pharmacology of acetylsalicylic acid. He considers the compound as a prodrug of the active metabolite salicylic acid. The first clinical studies by <i>Witthauer</i> and <i>Wolgemuth</i> are published the same year.
1899	Introduction of acetylsalicylic acid for the treatment of fever and pain under the trade name “Aspirin.”
Since 1899	Worldwide use of aspirin as a household remedy for treatment of fever, pain, and inflammation.
1945–1952	<i>Singer</i> describes a bleeding tendency after surgical interventions if aspirin was used for analgesic purposes. This observation is confirmed in other case reports. <i>Singer</i> explains this by a reduction of prothrombin levels.
1949–1950	<i>Gibson</i> reports of the positive results with aspirin for the treatment of anginal pain, according to a survey by 20 physicians. <i>Craven</i> , a general practitioner from Glendale (California), publishes shortly thereafter his first study on antithrombotic effects of aspirin. According to his data, daily administration of 650–1950 mg aspirin completely prevented myocardial infarctions in 400 male, medium-aged patients during an observation period of 2 years.

(Continued)

Table 1.1 (Continued)

Date	Event
1967–1968	<i>O'Brien, Zucker, Weiss,</i> and coworkers publish the first mechanistic studies on the antiplatelet activity of aspirin. Daily doses of 75 mg cause an inhibition of platelet function over several days. According to his data, <i>O'Brien</i> recommends a clinical trial with aspirin for thrombosis prevention in patients at elevated vascular risk.
1971	<i>Sir John Vane</i> detects the inhibition of prostaglandin synthesis by aspirin (and salicylate) and considers this as the mechanism of the anti-inflammatory action. This work and the later detection of prostacyclin are acknowledged with the Nobel Prize for medicine in 1982.
1971	<i>Brian Smith</i> and <i>Al Willis</i> , both working in <i>John Vane's</i> laboratory, detect the inhibition of prostaglandin synthesis by aspirin in platelets and explain its antiplatelet action by this property.
Since 1971	Systematic search for and development of cyclooxygenase inhibitors with prospective use as symptomatic anti-inflammatory analgesics.
1975	The group of <i>Philip Majerus</i> detects the acetylation of platelet cyclooxygenase by aspirin and explains by this mechanism the inhibition of thromboxane formation and platelet function.
1979	<i>Crandon</i> and <i>Isherwood</i> report that regular intake of aspirin in pregnancy reduces the risk of preeclampsia. <i>Beaufils</i> confirms this finding in high-risk patients in 1985 in a randomized study.
1983	Publication of the first placebo-controlled randomized double-blind trial (Veterans Administration Study) on prophylactic use of aspirin (324 mg/day) in men with acute coronary syndromes by <i>Lewis</i> and colleagues. The study shows a 50% reduction of the incidence of myocardial infarctions and death within an observation period of 3 months.
1988	Publication of the first clinical findings of a relationship between aspirin intake and prevention of colon cancer by <i>Gabriel Kune</i> and colleagues from Melbourne. In this retrospective, exploratory case–control study, regular (daily) use of aspirin reduced the risk of (incident) colon cancer by 40%. These findings were later confirmed and extended by <i>Michael Thun</i> and colleagues (1991) in a large prospective epidemiological trial (CPS-II Study) in the United States.
1988–1989	Publication of the first two prospective, placebo-controlled long-term trials on primary prevention of myocardial infarctions in apparently healthy men in the United States (USPHS) and the United Kingdom (BMDS), respectively. The results are controversial. The American study suggests a beneficial effect of aspirin on the prevention of a first myocardial infarction. <i>Charles Hennekens</i> (USPHS) receives the Aspirin Senior Award in 1999 for his significant contributions to the use of aspirin for prevention of atherothrombotic events.
1989	The ISIS-2 trial, a prospective, placebo-controlled randomized trial in patients with acute myocardial infarction, demonstrates a remarkable protective action of aspirin (162 mg/day), alone and a doubling of the effect in combination with streptokinase, on prevention of recurrent myocardial infarctions and death for an observation period of 5 weeks. This study leads to guideline recommendation of aspirin in secondary prevention.
1988–1990	<i>William Smith, David De Witt,</i> and colleagues demonstrate that the molecular mechanism of aspirin action is due to steric hindering of access of the substrate (arachidonic acid) to the enzyme (cyclooxygenase) and does not involve direct binding of the agent to the active center. <i>William Smith</i> receives the Aspirin Senior Award in 1997 for this and other major contributions to the better understanding of the molecular mechanism of aspirin action.

Table 1.1 (Continued)

Date	Event
1991	Publication of the prospective, randomized, placebo-controlled study (SALT trial) on low-dose (75 mg/day) aspirin in patients with transient ischemic attacks (TIA). The number of strokes, TIA, and myocardial infarctions are markedly reduced by aspirin, whereas the number of hemorrhagic infarctions is increased. The benefit–risk ratio is clearly in favor of prevention.
1991	<i>Kenneth Wu</i> and colleagues show inhibition of cytokine-induced expression of cyclooxygenase (-2) in human endothelial cells by aspirin and salicylate but not by indomethacin. This suggests salicylate-mediated inhibition of gene transcription. Later work of this group [33] identifies inhibition of binding of the C/EBP- $\beta$ transcription factor as (one) molecular mechanism of action.
1995	Patrick Loll, Daniel Picot, and R. Michael Garavito describe the crystal structure of COX-1 inactivated by an aspirin analogue. <i>Patrick Loll</i> receives the Aspirin Junior Award in 2000 for his significant contributions to this research.
1995	Jose Claria and <i>Charles Serhan</i> detect the generation of ATL by the interaction of acetylated COX-2 with the 5-lipoxygenase of white cells. The contributions of <i>Claria</i> , working in Serhan's group, to this research are acknowledged with the Young Researchers' Aspirin Award in 1996.
Since 1998	Search for new fields of clinical use of aspirin and aspirin-“like” drugs, including the prevention of progression of Alzheimer's disease.
2000	The Oxford group around <i>Sir Richard Peto</i> , <i>Rory Collins</i> , and <i>Peter Sleight</i> receives the Aspirin Senior Award for their outstanding contributions to developing and conducting meta-analyses in studies with antiplatelet drugs. The latest edition dates to 2002.
2005	Publication of the prospective, randomized, placebo-controlled Women's Health Study (WHS). The study demonstrates the usefulness of aspirin (100 mg each second day) for the prevention of atherothrombotic events in apparently healthy women during a 10-year observation period. There is significant protection from ischemic cerebral infarctions but not from myocardial infarctions. The protective action of aspirin increases with increasing vascular risk.
2006	The CHARISMA study compares low-dose aspirin (75–162 mg) alone and in combination with clopidogrel (75 mg) in primary prevention of vascular events in high-risk populations. Although the combination is useful in secondary prevention, similar to CAPRIE, the comedication of clopidogrel with aspirin did not reduce the vascular risk but increased bleeding in patients with risk factors but without preexisting event.
2010	?

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## 1.2 Chemistry

This chapter on chemistry and measurement of salicylates is focused on pharmaceutical aspects of aspirin and other salicylates. It includes a description of the physicochemical behavior of this class of compounds and methods for their measurement in biological media. This chapter is not written with the intention to provide a complete overview of all chemical and analytical aspects of salicylates but rather to inform about those issues that are relevant to the understanding of their pharmacology and toxicology in biological systems.

The first part of this chapter describes chemical structures and physicochemical properties of aspirin and other salicylates (Section 1.2.1). The physicochemical properties of salicylates are unique to this class of compounds, specifically the mesomeric structures of salicylate, eventually resulting in chelating properties and allowing incorporation of salicylate into the cell membrane phospholipids. This physicochemical property is of outstanding importance to understand the actions of salicylates on cellular energy metabolism, specifically the uncoupling of oxidative phosphorylation. Another aspect is the (poor) water solubility of aspirin and salicylic acid (in contrast to sodium salicylate), eventually resulting in local irritations of stomach mucosa after oral administration (Section 3.2.1). Finally, the particular crystal structure of aspirin and the recent discovery of two polymorphic forms, coexisting in one and the same aspirin crystal, is an issue of considerable pharmacological interest.

The second part of this chapter describes analytical methods of salicylate determination in biological media (Section 1.2.2). Several techniques are available for simultaneous measurement of aspirin and its major metabolites.

### 1.2.1 Structures and Chemical Properties of Salicylates

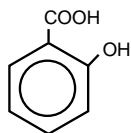
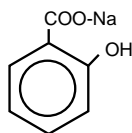
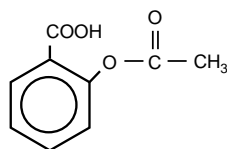
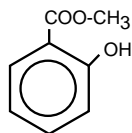
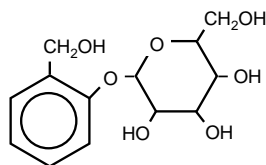
The glucoside salicin was the first active ingredient of the willow bark, which was isolated as a crystal-

line agent by Leroux in 1830 (Section 1.1.2). Leroux obtained 1 ounce (about 28.3 g) salicin from 3 pounds of willow bark (*Salix helix*) (cited after Ref. [67]). Salicin was later used by Piria as starting material for the preparation of salicylic acid (Section 1.1.2). Salicylic acid (*o*-hydroxybenzoic acid) is a relatively strong acid, having a  $pK_a$  of 2.9 and is poorly water soluble (0.2%). Its solubility can be considerably improved by converting the compound into the sodium salt, which is approximately 50% water soluble. Salicylates for systemic use are either esters with substitutions in the carboxyl group, such as methylsalicylate, or esters of organic acids with substitutions in the phenolic *o*-hydroxyl group, such as aspirin. Aspirin is the acetate ester of salicylic acid (Figure 1.7). The crystalline and molecular structure of aspirin has been elucidated [68, 69].

Computer calculations, however, have provided evidence for another even more stable crystalline form of aspirin with a close relationship with the already known form I [70]. Most recent experimental studies were able to confirm the real existence of form II and, in addition, showed that a polymorphism exists between these two forms. Importantly, the two different polymorphs can coexist within one and the same crystal [71, 72]. This new and unexpected finding with aspirin as the first compound to show this unique property raises a number of principal questions regarding the definition of crystal polymorphism, which is outside the further discussion about pharmacological and toxicological properties of the compound. There are also the legal issues that are important in this context – each polymorph of each compound can be patented separately.

#### 1.2.1.1 Salicylates in Clinical Use

**Salicylic Acid** Salicylic acid (molecular weight 138.1) in the form of sodium salt (molecular weight 160.1) was the first purely synthetic salicylate in clinical use (Section 1.1.3). It is no more used for systemic internal administration because of its unpleasant, sweetish taste and irritation of the stomach mucosa. Not only as an antipyretic analgesic but also as an anti-inflammatory drug, it has been replaced by better tolerable agents, such as

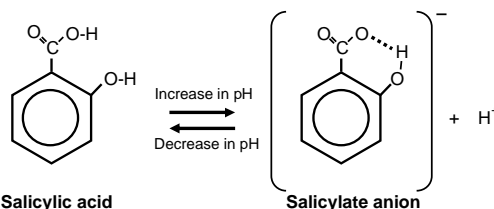
**Salicylic acid****Sodium salicylate****Methyl salicylate****Acetylsalicylic acid****Salicin****(2-[Hydroxymethyl]phenyl-β-D-glycoside)****Figure 1.7** Chemical structures of selected salicylates.

aspirin or the structurally different, more potent anti-inflammatory compounds, NSAID (Section 4.2.2) and acetaminophen (paracetamol). Nevertheless, salicylate is still being used as an external medication, for example, in ointments because of its antiseptic and keratolytic properties.

Despite its disappearance from internal medicine, the pharmaceutical and biological properties of salicylate are of considerable pharmacological interest because this compound is the primary metabolite of aspirin and responsible for many of its biological actions including salicylate poisoning (Section 3.1.1). Salicylate shows a peculiar physicochemical behavior because of the formation of a ring structure by hydrogen bridging. This requires a hydroxy group in a close neighborhood of the carboxyl group and is only seen with the *o*-hydroxybenzoic acid salicylic acid (Figure 1.8) but not with its *m*- and *p*-analogues. The *o*-position of the hydroxyl group facilitates the release of a proton with decreasing pH by increasing the mesomerism of the resulting anion. These properties are biologically relevant for the protonophoric actions of salicylates in the uncoupling of oxidative phosphorylation by eliminating the impermeability of cell

membranes to protons (Section 2.2.3). In addition, they help understand the local irritation of the stomach mucosa subsequent to direct contact with the compound and its incorporation into mucosal cells (Section 3.2.1). The *m*- and *p*-hydroxy analogues of benzoic acid do not share these properties with salicylate and are biologically largely inactive.

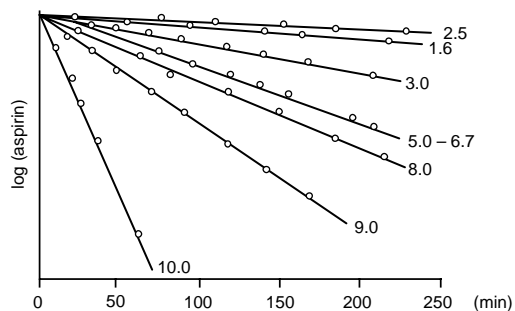
Structure–activity studies of 80 salicylate-type compounds for uncoupling oxidative phosphorylation in isolated mitochondria showed that the essential pharmacophore for this activity is a compound with a negatively charged (carboxyl) group at the *o*-position, that is, acetylsalicylate. The *m*- and *p*-hydroxybenzoate analogues of salicylic acid failed to do so. This suggested

**Figure 1.8** pH-dependent equilibrium of ionized and nonionized forms of salicylate.

the *o*-position of the hydroxyl group as an essential steric requirement for this activity. Mechanistically, this was explained by the unique proton bridging between the oxygen of the carboxyl group and the proton in the hydroxy group, allowing for a mesomeric state that promotes a nondissociated configuration and facilitates tissue penetration [73].

**Acetylsalicylic Acid** Acetylsalicylic acid (molecular weight 180.2) or aspirin is the acetate ester of salicylic acid. The pharmacological properties are similar to those of salicylate. However, aspirin also has activities of its own, which are added by the reactive acetate group – the (nonselective) acetylation of cellular targets, including proteins and DNA. This results in biological effects that are not shared by salicylate. Examples are the inhibition of platelet function by irreversible acetylation of COX-1 and the acetylation of COX-2 with subsequent formation of 15-(R)-HETE and generation of ATL by white-cell 5-lipoxygenases (Section 2.2.1).

Aspirin is a white powder with a pleasant acidic taste. The compound is poorly soluble in water (0.3%) and somewhat better soluble in ethanol (20%). The solubility in aqueous media depends on pH. It amounts to only 60 µg/ml at pH 2, but increases dramatically with increasing pH (Figure 1.9). The solubility in aqueous media is also markedly improved after its conversion into the sodium salt, specifically at acidic pH (Table 1.2).



**Figure 1.9** pH-dependent hydrolysis of aspirin (1.5 mM) in aqueous solution at 42 °C. Note the high stability (poor solubility) of aspirin at acidic pH and the significant increase in solubility (rapid degradation) at alkaline pH [74].

**Table 1.2** Dissolution rates of various salicylates in 0.1 N hydrochloric acid (modified after Levy and Leonards, 1966).

Compound	Dissolution rate (units at current stirring and 37 °C)	
	Absorption	Release
Salicylic acid	38	1.0
Acetylsalicylic acid (aspirin)	49	1.3
Sodium acetylsalicylate	4900	130.0
Sodium salicylate	5200	140.0

This pH-dependency of solubility of aspirin is one of the reasons for its irritation of stomach mucosa in the acidic gastric juice (Section 3.2.1) as well as the increasing absorption in the upper intestine because of markedly improved solubility that also dominates the increased dissociation rate.

**Methylsalicylate** Methylsalicylate is the active ingredient of wintergreen oil from the American teaberry (*G. procumbens*). This oil was used as a natural source of salicylates since the early nineteenth century because it contained 98% methylsalicylate, the active constituent of numerous drug combinations for external use in rheumatic diseases. It is also of toxicological interest because of its much higher toxicity in comparison to other salicylates. Particularly dangerous is the erroneous ingestion (by children) of methylsalicylate-containing ointments and other products for external use (Section 3.1.1).

### 1.2.1.2 Aspirin Formulations

Several galenic preparations of aspirin have been developed for practical use with the intention to improve the solubility and stability of the compound and to reduce or even avoid gastric irritations. The first property is particularly relevant if short-term or immediate action of aspirin is desired, for example, in the treatment of migraine or tension-related headache. Alternatively, aspirin can be administered intravenously or orally as the well water-soluble lysine salt. This is of particular

interest if high levels of unmetabolized aspirin have to be obtained in the systemic circulation, for example, to immediately block platelet function in the acute coronary syndromes (Section 4.1.1). Another approach is an enteric-coated formulation, predominantly for long-term use. A detailed discussion of these and other formulations in clinical use is found elsewhere (Section 2.1.1).

**Generics** A frequently discussed issue in clinical pharmacology is whether all formulations of all manufacturers containing the same active ingredient at the same dose are bioequivalent – with the consequence of preferential use of the cheapest formulation. In the case of salicylates, this has been refused already many years ago [74]. A more recent study additionally suggested that even aspirin analogues that passed pharmaceutical *in vitro* dissolution specifications may not be bioequivalent *in vivo*

[75]. Moreover, a comparative study of selected aspirin formulations in Germany has found large differences regarding the pharmaceutical quality of aspirin-containing monoprparations with suggested use as antipyretic analgesics.

In 1986, a comparison of all pharmaceutical OTC formulations of aspirin was performed. Included were all products containing aspirin as active ingredient, which were on the German market for suggested use as antipyretic analgesics. Only tablets and no other galenic preparations or tablets for other symptoms were included.

All 11 tablets fulfilled general requirements regarding, for example, the content of the active ingredient. However, according to the authors, marked and unacceptable differences existed with respect to the individual *in vitro* release kinetics. These criteria were determined according to an US Standard and were not met by 5 out of the 11 preparations tested (Table 1.3) [76].

**Table 1.3** *In vitro* release kinetics of ASA under standard conditions from commercially available ASA preparations in Germany [76].

ASA preparation	Declared ASA content (mg)	Percentage of declaration found	ASA release kinetics (% in 30 min ± SD)
Acetylin	500	98.9	89.6 ± 2.8
Aspirin	500	99.4	100.7 ± 1.0
Aspirin junior	100	102.9	103.5 ± 2.7
Aspro	320	98.1	96.4 ± 4.7
Ass 500 Dolormin	500	98.0	77.2 ± 9.2
ASS-Dura			
Ch.-B. 18613	500	98.2	65.4 ± 16.5
Ch.-B. 074035	500	102.9	72.4 ± 9.5
ASS-Fridetten			
Ch.-B. 019026	500	98.3	68.8 ± 10.0
Ch.-B. 020047	500	100.1	75.9 ± 5.7
ASS-ratiopharm	500	96.6	89.3 ± 9.3
ASS-Woelm	500	100.9	77.9 ± 8.8
Temagin ASS 600			
Ch.-B. 212142	600	100.9	50.2 ± 11.4
Ch.-B. 212143	600	100.4	44.0 ± 9.9
Trineral	600	99.2	90.8 ± 3.9

According to predefined quality standards, the content of the active ingredient should be 95–105% of declaration and at least 80% of the compound should be released within 30 min under the conditions chosen.

The authors repeated this study 2 years later on 62 different aspirin preparations. They found that of the tested formulations, 20 (!) still did not meet the quality standards mentioned above [77]. Thus,

despite containing ASA as the active ingredient at identical amounts, not all aspirin preparations might be the same in terms of bioavailability of the compound.

### Summary

Aspirin and its main metabolite salicylic acid are poorly water soluble at acidic and neutral pH. The solubility increases markedly in alkaline pH and is more than 100-fold higher for the sodium salts as compared to the free acids. This is also valid for strong acidic pH.

Salicylates, in contrast to aspirin, have unique physicochemical properties. These are caused by the close steric neighborhood of the acetate hydroxyl group to the carboxyl group. This allows the formation of a chelate ring structure and facilitates the release of protons.

The major functional consequence is the action of salicylate as protonophore, for example, in mitochondrial membranes, to uncouple oxidative phosphorylation because of the abolition of the membrane impermeability to protons (Section 2.2.3). Neither aspirin nor other salicylates exhibit comparable physicochemical properties.

Aspirin is commercially available in many different galenic formulations. *In vitro* studies on bioequivalence provided different results for different formulations. However, the functional consequences for clinical use have not been studied sufficiently.

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### 1.2.2

#### Determination of Salicylates

Measurement of salicylates in biological fluids, that is, mainly plasma and urine, is of interest for several purposes. The purpose also determines the selection of the method. Most frequent is the control of plasma levels to verify that the plasma concentrations are within the therapeutic range. Determination of plasma levels is also necessary in the case of intoxication and for controlling the efficacy of detoxification procedures. Plasma or urinary levels of salicylates allow checking patients' compliance, an important issue for long-term aspirin use in cardiovascular prophylaxis and a frequent explanation of the so-called aspirin resistance (Section 4.1.6). Finally, measurements are of interest to study the pharmacokinetics of salicylates in research, in particular, drug metabolism and interactions.

The therapeutic plasma levels of salicylate differ, depending on the indication. They are in the range of 100–200 µg/ml at anti-inflammatory doses and 50–100 µg/ml for analgetic purposes (see Figure 2.23). The plasma levels of unmetabolized ASA are approximately one order of magnitude lower. Thus, assay methods should be sensitive enough to detect amounts above 1 µg/ml [78]. In most cases, no separate determination of aspirin and salicylate is necessary because there is a rapid and complete conversion of aspirin into salicylate *in vivo*.

##### 1.2.2.1 Gas–Liquid Chromatography

Gas–liquid chromatography (GLC) is the reference standard. The technique allows separate determination of ASA, salicylic acid, and their metabolites. The detection limit is 1 µg/ml.

##### 1.2.2.2 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is an alternative to GLC but more complex and time consuming. Reverse-phase HPLC techniques with photometric detection are the methods of choice [79]. This has the advantage that the complete spectrum of aspirin and its metabolites can be measured simultaneously. However, one major problem is associated with this type of assay. This

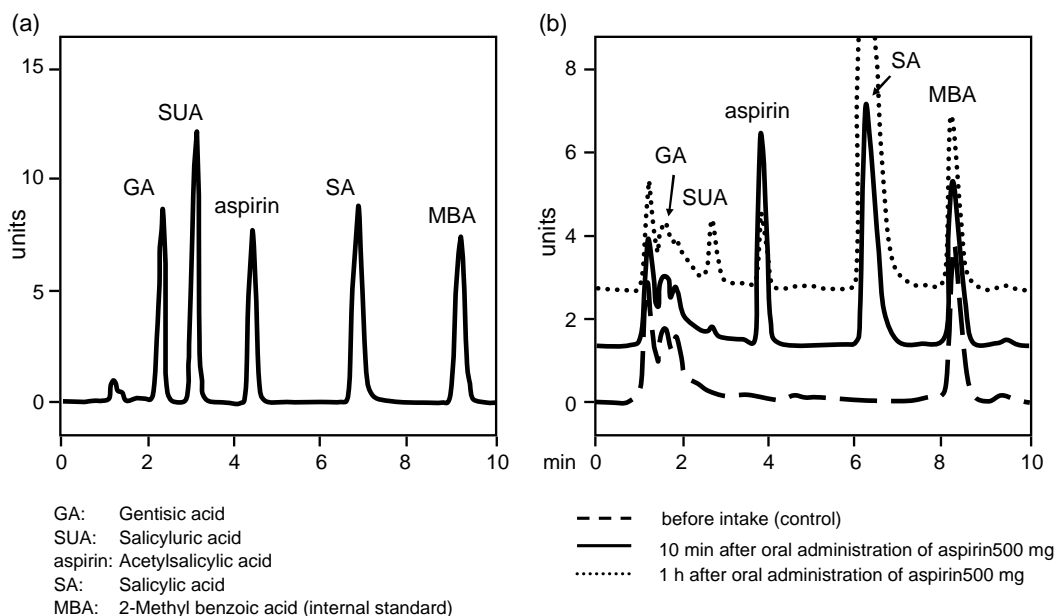
is the spontaneous hydrolysis of aspirin to salicylate in protic solvents, including water, methanol, and plasma (Section 2.1.1). Thus, some degradation of aspirin may occur *ex vivo*. A modification of this technique for human plasma, including extraction of salicylates in organic solvents, allows simultaneous determination of aspirin and its metabolites down to the levels of 100 ng/ml with an interassay variation of less than 10% (Figure 1.10). This technique combines simplicity in sample treatment with stability of aspirin over several days (!) without significant decomposition [80].

##### 1.2.2.3 Spectrophotometry

Spectrophotometry is the earliest and most widely used method for measuring serum salicylate levels. The classical assays are colorimetric assays, taking advantage from the intense red color of salicylate/Fe<sup>3+</sup> complexes. The technology is simple and particularly suitable for compliance measurements.

**Trinder Method** The Trinder method [81] is a colorimetric test where salicylic acid is determined by measuring the absorbance of the ferric ion–salicylate complex after total serum protein is precipitated by mercuric chloride and allowed to react with ferric iron supplied by ferric nitrate. This method involves the generation of a complex between salicylate and ferric ion.  $A_{\max}$  of the ferric complex is 540 nm. Quantification is done by measuring light absorbance at this wavelength by a spectrophotometer.

The Trinder method is simple, inexpensive, rapid, and very reliable. Spectrophotometry lowers the detection limit to about 100 µg/ml. This is sufficient for therapeutic and toxic purposes. However, the Trinder method measures salicylate rather than ASA. (False) Positive results may be obtained with salicylamide or methylsalicylate. Conversely, the method can also be used to measure these compounds, for example, in the case of poisoning. The Trinder test is also rather nonspecific and sensitive to a large number of other acids and amines [82]. This also includes compounds and their metabolites, which are increased in patients with Reye-like symptoms because of hepatic (metabolic) failure [83]



**Figure 1.10** Chromatograms of a standard mixture of acetylsalicylic acid and major metabolites (50 ng each) (a) and plasma levels of a volunteer before, 10 min after, and 1 h after oral administration of 500 mg aspirin (b) (modified after Ref. [80]).

(Section 3.3.3). More recently, Morris *et al.* [84] have described a two-step colorimetric method that, however, so far has only been used in the research.

**Second-Derivative Synchronous Fluorescence Spectrometry** Another method that allows simultaneous determination of ASA and its major metabolites in one assay is second-derivative synchro-

nous fluorescence spectrometry (SDSFS) [85]. This method appears to be the first nonchromatographic technology for the simultaneous determination of aspirin and its major metabolites in one single-serum sample. The technique is not sensitive to several other drugs, found frequently in the sera of healthy subjects (antipyrine, ibuprofen, indomethacin, theophylline, and others).

### Summary

Several methods are available to determine aspirin and its major metabolites in biological fluids, including plasma (serum), liquor, synovial fluid, and urine. Most of them have the necessary sensitivity (detection limit 1  $\mu\text{g}/\text{ml}$  or less).

HPLC separation and subsequent identification of the spots by appropriate standards is the most frequently used technology. Advantages are the simplicity and reproducibility of the method, a high sensitivity (detection limit about 100 ng/ml), and the possibility of simultaneous deter-

mination of several aspirin metabolites together with aspirin itself in one sample. Disadvantages of this and some other technologies include the spontaneous (pH-dependent) and enzymatic hydrolysis of aspirin. However, this problem can be solved by appropriate sample processing.

The Trinder method, a colorimetric assay, determines salicylate and is also useful and simple, though less sensitive. It exhibits a number of cross-reactions with other compounds, which might become relevant, for example, in hepatic failure. GC/MS is clearly the most reliable technology. However, it needs expensive equipment and experienced investigators.

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