The identification of salicylates as normal constituents of serum: a link between diet and health?

John R Paterson, Claire Blacklock, Graham Campbell, David Wiles, James R Lawrence

Abstract

**Aim**—To examine sera for the presence of salicylic acid and 2,3- and 2,5-dihydroxybenzoic acids (2,3-DHBA, and 2,5-DHBA), in individuals not taking salicylate drugs.

**Methods**—Extracts of acidified serum samples were analysed by high performance liquid chromatography with electrochemical detection. The chromatographic conditions were altered, and the retention times of the unknown compounds compared against authentic salicylic acid, 2,3-DHBA, and 2,5-DHBA. Serum samples (some spiked with salicylic acid) were incubated with salicylate hydroxylase and analyses undertaken. An extract of acidified serum was derivatised using N-methyl-N-trimethylsilyl trifluoroacetamide and the salicylic acid derivative identified by gas chromatography—mass spectrometry.

**Results**—Salicylic acid, 2,3-DHBA, and 2,5-DHBA were identified as being normal constituents of serum.

**Conclusions**—Salicylic acid, 2,3-DHBA, and 2,5-DHBA possess anti-inflammatory properties. The finding that these compounds are present as normal constituents of serum, possibly arising from diet, raises important questions as to their role in the promotion of health.

(J Clin Pathol 1998; 51:502–505)

Keywords: salicylic acid; 2,3- and 2,5-dihydroxybenzoic acids; normal serum constituents; diet
To carry out chromatography of 2,3-DHBA and 2,5-DHBA, the Eox was +0.65 V and gradient elution was used: 0–6 minutes with 100% citrate buffer (0.03 mol/litre), pH 5.25 (by addition of glacial acetic acid), and 6–15 minutes in the same mobile phase with methanol 28.6% vol/vol. Chromatographic conditions were altered by changing the mobile phase to pH 4.75.

### INCUBATION OF SERUM WITH SALICYLATE HYDROXYLASE

Portions of serum (0.5 ml) from five different subjects were incubated with reduced nicotinamide adenine dinucleotide (β-NADH; 146 µmol/litre) and salicylate hydroxylase (0.48 units, obtained from *Pseudomonas* sp; Sigma, Poole, Dorset, UK) in a final volume of 1.5 ml with phosphate buffer (0.03 mol/litre), pH 7.62. Control reaction mixtures contained no salicylate hydroxylase or were spiked with salicylic acid (final concentrations 5 µmol/litre). Reaction mixtures were incubated at 30°C in a shaking waterbath for 15 minutes before extraction as described above.

### SERUM EXTRACTION, DERIVATISATION, AND ANALYSIS BY GC-MS

To an aqueous solution of salicylic acid (10 µmol/litre, 300 µl volume) was added HCl (100 µl, 1.0 mol/litre) and the resultant solution extracted twice with ethyl acetate (0.5 ml). The combined extracts were evaporated to dryness at 70°C under oxygen-free nitrogen and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; 300 µl) added. The reaction mixture was vortexed for 30 seconds and then heated at 70°C for 20 minutes to prepare the trimethylsilyl (TMS) derivative of salicylic acid.

To serum (7.5 ml) from one individual was added EDTA (final concentration 100 µmol/litre) and HCl (1.0 mol/litre to bring to pH 2.0) and the resultant mixture extracted twice with ethyl acetate (15 ml). The combined extracts were evaporated to dryness at 70°C under oxygen-free nitrogen and MSTFA (50 µl) added; the reaction mixture then treated in the same way as the salicylic acid standard above.

The derivatised extracts of salicylic acid and serum were transferred to sample vials for gas chromatography–mass spectrometry (GC-MS) analysis (Fisons, Manchester, UK: MD 800 mass spectrometer, GC 8000 series, and AS 800 autosampler, equipped with a 30 m, 0.25 mm DB-5 capillary column). A 1 µl sample was injected using a splitless injection port and a helium flow rate of 1 ml/min. The GC column temperature was initially set at 55°C for one minute, increasing by 30°C a minute to 265°C. The mass spectrometer was set at an electron energy of 70 eV and full scan mode (40.00 to 400.0 mass range).

## Results

Chromatography of the extracted sera from six individuals revealed the presence of an unknown substance, in each case having a similar retention time (t_r) to that of authentic salicylic acid.
Figure 2 HPLC chromatograms of an extract of (A) blank serum and (B) an aqueous mixture of 2,3- and 2,5-DHBA.

Table 2: Effect of changing the mobile phase pH on the retention time (t_r) of unknown substances present in serum and 2,3- and 2,5-DHBA

<table>
<thead>
<tr>
<th>Mobile phase pH</th>
<th>Aqueous standard 2,3-DHBA (t_r min)</th>
<th>Unknown substance (t_r min)</th>
<th>Aqueous standard 2,5-DHBA (t_r min)</th>
<th>Unknown substance (t_r min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.25</td>
<td>8.00</td>
<td>8.01</td>
<td>9.39</td>
<td>9.42</td>
</tr>
<tr>
<td>4.75</td>
<td>8.30</td>
<td>8.29</td>
<td>9.05</td>
<td>9.05</td>
</tr>
</tbody>
</table>

Discussion

We have found three different salicylates—salicylic acid, 2,3-DHBA, and 2,5-DHBA—to be present as normal constituents of serum. The previously unknown substances we had observed in chromatograms behaved in an identical fashion to the authentic salicylate compounds when chromatographic conditions were changed. Although similar changes in chromatographic parameters between an unknown substance and an authentic compound are not absolute proof that they are identical, the similarities described above, supported by the evidence obtained from the salicylate hydroxylase and GC-MS experiments, indicates to us that salicylic acid is present as a normal constituent of serum in “aspirin-free” individuals. The serum analysis was difficult because salicylic acid was present in low concentration and because of potential interference from MSTFA in the GC-MS experiments. There is, however, no serum sample analysed so far (n = 60) that has not contained salicylic acid. Examination of the chromatograms of blank serum or plasma from published HPLC methods of salicylic acid analysis reveals also that an unknown substance is present with a t_r similar to salicylic acid, but which is not commented upon in the respective papers. In addition, Ruffin et al reported salicylic acid in the plasma from 17 of 53 subjects at baseline, before aspirin intake, in a study examining the effect of aspirin on mucosal prostaglandins. No information was given as to how they identified the compound as being salicylic acid.

We are currently developing a method for quantitation of the naturally occurring salicylates and have observed significant differences between individuals. It is possible that diet is the source of salicylic acid since it is
found in fruits, berries, vegetables, herbs, and spices. Salicylic acid is metabolised in vivo to various compounds, including 2,3-DHBA and 2,5-DHBA, although these salicylates may also be present in our diet since they are known to be present in wine. Recently, from the measurement of salicylic acid in urine, the daily dietary intake of salicylic acid was estimated to be of the order of a few milligrams. The authors considered this intake to be too low to affect the risk of coronary heart disease or colon cancer “even if most of this would be in the form of acetylsalicylate” (aspirin). However, the dose of aspirin required to help prevent colorectal cancer is unknown, as is the minimum dose of aspirin effective in protecting the vasculature. The anti-platelet effect of aspirin is the major reason for prescribing the drug in cardiovascular disease, although recent work suggests that the anti-inflammatory action may also be important. It is possible that the anti-inflammatory action and chemopreventive action in colorectal cancer occurs by the same mechanism. In inflammation and colorectal cancer, cyclooxygenase (COX) 2, an enzyme catalysing the formation of prostanoid compounds, is induced by various stimuli including cytokines and growth factors, unlike the constitutively expressed COX 1 which is found in platelets. Aspirin is more potent than salicylate at inhibiting prostaglandin synthesis in vitro through its effects on COX activity, but the two compounds are considered to be equipotent as anti-inflammatory agents. In vivo, aspirin probably acts as an anti-inflammatory produg, the active component being salicylic acid. Salicylic acid has been shown to inhibit the induction of COX activity by interleukin -1, an effect observed at nanomolar concentrations of salicylic acid. 2,3- and 2,5-DHBA have also been shown to inhibit COX activity, to interfere with the cytokine production, and inhibit the formation of prostaglandin E2 in vitro. We believe that our finding of salicylic acid, 2,3-DHBA, and 2,5-DHBA as normal constituents of serum may provide a link between diet and the prevention of colorectal cancer and atherosclerosis. A diet which is rich in fruit and vegetables helps prevent colorectal cancer and atherosclerosis, and although various dietary components have been examined as possible candidates to explain the benefits of such a diet, no individual responsible component has been identified, especially in the prevention of cancer. We are currently investigating the relation between diet and serum salicylate concentrations in “aspirin-free” patients.

We thank the late Mr G G L Willeco, Dumfries and Galloway Acute and Maternity Hospitals NHS Trust and the Chest, Heart and Stroke Association (Scotland) for financial assistance, and acknowledge the valuable advice of Dr A B Graham, Strathclyde University, Glasgow.

13 Muller CJ, Fugeibang KC. Take two glasses of wine and see me in the morning. Lancet 1994;343:1428–9.