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-trimethylsilyltrifluoroacetamide 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. Salicylates, initially obtained from the bark of the willow tree, have been used in medicine to treat inflammatory conditions for many centuries. Within the past century, aspirin has been found to benefit patients not only as an anti-inflammatory drug, but also in preventing strokes, myocardial infarction, and death in patients with atherosclerosis. More recently, aspirin has been found to have a chemopreventive action against colorectal cancer, a property shared also by other non-steroidal anti-inflammatory drugs. Aspirin is rapidly hydrolysed to salicylic acid in vivo, and its anti-inflammatory action is believed to be the result of the formation and action of salicylic acid. Salicylic acid has also been found to induce cell cycle arrest and apoptosis in cultured colorectal tumour cells, indicating that the chemopreventive property of aspirin may act through salicylic acid formation as well.

While investigating the use of low dose aspirin as an aromatic probe to measure hydroxyl free radicals (by assessing the hydroxylation of salicylic acid, with resulting formation of 2,3- and 2,5-dihydroxybenzoic acid (2,3- and 2,5-DHBA)), we observed the presence of substances which had identical retention times to salicylic acid, 2,3-DHBA, and 2,5-DHBA in serum extracts from subjects not taking aspirin. After excluding the possibility of contamination, we investigated whether these substances were authentic salicylic acid, 2,3-DHBA, and 2,5-DHBA.

Methods

BLOOD SAMPLES

Blood samples were obtained from healthy volunteers who were not taking any drugs, nor had they been using other preparations which contain salicylates, for example mouthwashes. Whole blood was allowed to coagulate before separating the serum by centrifugation (2000 g for 10 minutes). Aliquots of serum were stored at −28°C.

SALICYLATE EXTRACTION AND HPLC ANALYSIS

Ethylenediaminetetra-acetic acid (EDTA; final serum concentration 100 µmol/litre) was added to portions of serum (0.5 ml) which were then acidified to pH 2.0 by the addition of HCl (1.0 mol/litre), and ethyl acetate (2.0 ml) then added. The extraction tubes were shaken for 15 minutes, centrifuged at 2000 g, and the organic phase removed and evaporated to dryness at 70°C under oxygen-free nitrogen. The extraction process was repeated and the combined extracts reconstituted in high performance liquid chromatography (HPLC) mobile phase—0.50 ml, citrate buffer (0.03 mol/litre) brought to pH 4.0 with the addition of glacial acetic acid, methanol (29.6% vol/vol), and EDTA (100 µmol/litre). A Jasco (Great Dunmow, Essex, UK) PU-980 HPLC pump and LG 980-02 ternary gradient unit were used to deliver mobile phase at 1.0 ml/min through an Apex ODS 5 µm column (Jones Chromatography, Hengoed, Shropshire, UK). Amperometric detection was carried out using an Antec Decade detector (Presearch, Hitchin, Herts, UK) with an oxidising potential (Eox) of +1.1 V. Fifty microlitres of the reconstituted extract were injected with a run time of 30 minutes. Chromatographic conditions were altered by reducing the methanol concentration and by varying the Eox of the electrochemical detec-
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 buVer (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 (\(\beta\) NADH; 146 \(\mu\)mol/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 buVer (0.03 mol/litre), pH 7.62. Control reaction mixtures contained no salicylate hydroxylase or were spiked with salicylic acid (final concentrations 5 \(\mu\)mol/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 \(\mu\)mol/litre, 300 \(\mu\)l volume) was added HCl (100 \(\mu\)l, 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-trimethylsilyl trifluoroacetamide (MSTFA; 300 \(\mu\)l) 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 \(\mu\)mol/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 \(\mu\)l) 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 \(\mu\)l 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 as normal constituents of serum.

![Figure 1](http://jcp.bmj.com/)

**Figure 1** HPLC chromatograms of extracts of reaction mixtures: (A) blank serum and NADH; (B) blank serum, NADH, and salicylate hydroxylase; (C) blank serum, NADH, salicylic acid, and salicylate hydroxylase.

<table>
<thead>
<tr>
<th>Methanol (% vol/vol)</th>
<th>SA ((t_r) min)</th>
<th>Serum spiked with SA ((t_r) min)</th>
<th>Blank serum: unknown substance ((t_r) min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.0</td>
<td>9.05</td>
<td>9.00</td>
<td>8.95</td>
</tr>
<tr>
<td>20.6</td>
<td>8.79</td>
<td>8.76</td>
<td>8.79</td>
</tr>
<tr>
<td>23.0</td>
<td>8.14</td>
<td>8.10</td>
<td>8.09</td>
</tr>
<tr>
<td>28.6</td>
<td>7.30</td>
<td>7.26</td>
<td>7.22</td>
</tr>
</tbody>
</table>
results were obtained for all five individuals investigated.

The TMS derivative of salicylic acid was found to have a $t_r$ of 6.461 minutes when analysed by GC-MS. Examination of the chromatogram obtained from the derivatised serum extract revealed substances with overlapping $t_r$ values of 6.421 and 6.471 minutes. With GC-MS analysis of MSTFA in hexane, compounds with $t_r$ values of 6.431, 6.481, and 6.522 minutes, respectively, were found. Total ion chromatograms showed the presence of an abundant ion at $m/z$ 267 in the serum extract and in MSTFA; however, the best fit for the fragmentation pattern at 6.441 minutes in the serum extract was TMS, salicylic acid (TMS, salicylic acid molecular ion minus methyl). Various siloxane compounds best fitted the MSTFA total ion chromatogram.

**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 sources by 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

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**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>

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**Figure 2** HPLC chromatograms of an extract of (A) blank serum and (B) an aqueous mixture of 2,3- and 2,5-DHBA.
Salicylates as normal constituents of serum

We thank the late Mr G L Willock, Drumfries 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, Fuglsang KC. Take two glasses of wine and see me in the morning. Lancet 1994;343:1428–9.
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