Biochem. J. (1986) 237, 499–504 (Printed in Great Britain)

Aromatic hydroxylation as a potential measure of hydroxyl-radical formation *in vivo*

Identification of hydroxylated derivatives of salicylate in human body fluids

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Attack by 'OH radicals, generated by a Fenton system, upon salicylate produces 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate as major products and catechol as a minor product. H.p.l.c. separation combined with electrochemical detection was used to identify and quantify 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate in human plasma and synovial fluid. We propose that conversion of salicylate into 2,3-dihydroxybenzoate, or of other aromatic compounds into specific hydroxylated products, may be a useful assay for 'OH formation in the human body.

INTRODUCTION

Oxygen-derived species such as $O_2^{\cdot-}$ and H_2O_2 have been implicated as damaging agents in the action of many toxins and in a number of diseases [1, 2]. The possibility that much of the toxicity produced by increased $O_2^{\cdot-}$ and H_2O_2 generation is mediated by metal-ion-dependent formation of the highly reactive 'OH radical has also been discussed in detail [3–5]. Although much circumstantial evidence supports the biological relevance of the iron-ion-catalysed formation of 'OH radical from $O_2^{\cdot-}$ and and H_2O_2 [3, 4, 6], there has been as yet no direct demonstration that 'OH radical is formed *in vivo*. The inflamed rheumatoid joint, in which conditions are proposed to be ideal for 'OH generation [4], seems a good place to look.

The 'OH radical is so highly reactive that proving its formation *in vivo* will be correspondingly difficult. Spin traps such as 5,5-dimethylpyrroline *N*-oxide (DMPO) do not seem promising for the detection of 'OH *in vivo* [7], nor does the conversion of 4-methylthio-2-oxobutyrate into ethene [19]. Hence we have attempted to adapt our technique for measuring 'OH radicals *in vitro*, aromatic hydroxylation [8–10], for use *in vivo*. Aromatic compounds react with high rate constants with 'OH, often to form a specific set of hydroxylated products [11, 12]. If an aromatic compound can be safely administered to humans in doses that produce concentrations in body fluids sufficient to scavenge 'OH, then observation of those products expected from attack of 'OH on the aromatic compound would be good evidence that 'OH is being formed *in vivo*, provided that the same products are not formed by enzymic hydroxylating systems.

Aspirin (*O*-acetylsalicylate) is still sometimes used in the treatment of rheumatoid arthritis. It is rapidly hydrolysed to salicylate *in vivo* [14, 15]. Attack of 'OH upon salicylate produces two products that have not been reported as normal products of enzymic salicylate metabolism, namely 2,3-dihydroxybenzoate and, to a much smaller extent, catechol [12, 15, 16]. We have therefore developed techniques to identify these products in human body fluids.

MATERIALS AND METHODS

Reagents

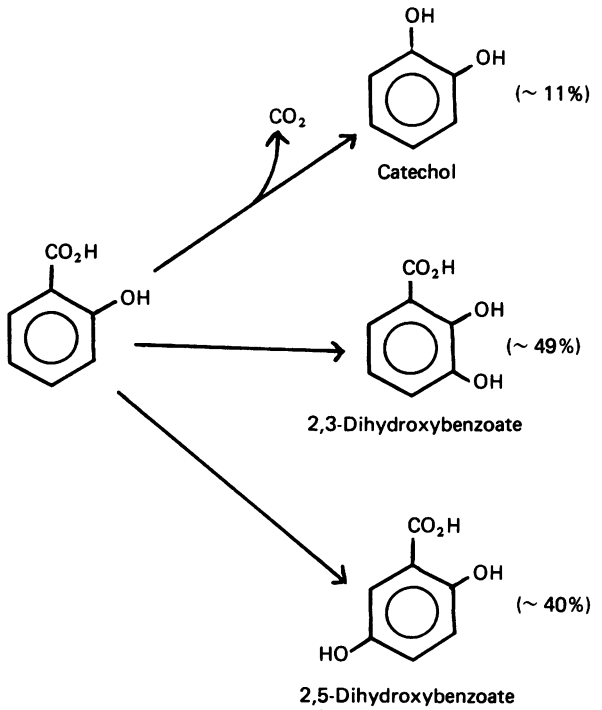
H.p.l.c.-grade solvents were obtained from BDH Chemicals, and aromatic compounds from Aldrich Chemical Co. Standard solutions of 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, salicylate, catechol and resorcinol were made up in h.p.l.c.-grade water containing 50 mM-HCl, to a maximum concentration of 100 μM. Standard solutions of salicylurate were in h.p.l.c.-grade water alone. Storage of solutions at 4 °C for periods of 1 week or less did not affect their concentrations; solutions were allowed to reach room temperature and thoroughly mixed before use.

Body fluids

Plasma and knee-joint synovial fluid were drawn into heparinized tubes for genuine therapeutic purposes from patients with moderately severe rheumatoid arthritis according to the criteria of the American Rheumatism Association. Patient A had been on long-term aspirin treatment (600 mg four times daily for 3 months). Aspirin was stopped for 5 days, then restarted, and the samples were taken 5 days later. Patients B and C each took a single 600 mg dose of aspirin. Synovial-fluid samples were centrifuged immediately and the supernatants used for study. Control samples of plasma were drawn from healthy adult male volunteers, not age-matched to the rheumatoid patients.

H.p.l.c.

H.p.l.c. was carried out on an HPLC Technology Ltd. or a Jones Chromatography Ltd. Spherisorb 5 ODS reverse-phase column (25 cm × 4.6 mm). The mobile phase (composition described in Figure legends) was sparged continuously with He gas during elution. Detection was by an EDT LCA15 electrochemical detector equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode. The injection loop was cleaned at least three times with 0.1 ml of h.p.l.c.-grade water after each injection.



Scheme 1. Products of the attack by 'OH radicals on the salicylate molecule

Normal metabolites of aspirin have been reported to be salicylate, 2,5-dihydroxybenzoate (gentisate) and salicylurate [13–15]. 2,3-Dihydroxybenzoate [15] and catechol have not been reported as salicylate metabolites. The percentages quoted are percentages of total hydroxylated products formed. Generation of 'OH was carried out in 20 mM-phosphate buffer, pH 7.4, as described in ref. [12].

RESULTS

Mixing an Fe^{2+} salt and H_2O_2 in phosphate buffer at pH 7.4 produces 'OH radicals. If salicylate is included in the reaction mixture under these physiological conditions, three products are formed (Scheme 1). 2,5-Dihydroxybenzoate (gentisate) has been reported to be an enzyme-produced metabolite of salicylate in humans, but 2,3-dihydroxybenzoate and catechol have not [13–15]. Since Aspirin (*O*-acetylsalicylate) is rapidly hydrolysed to salicylate *in vivo*, we decided to try and measure the concentration of the above three products in plasma and knee-joint synovial fluid from patients with active rheumatoid arthritis who had taken aspirin, and to compare them with the concentrations in the plasma of healthy human volunteers taking aspirin. No single chromatographic method was found to be adequate to identify conclusively all three products in human body fluids, and so three different methods have been developed.

Method A

In this method samples were extracted with diethyl ether, the h.p.l.c. solvent was citrate/acetate buffer and the detector potential (usually 0.60–0.68 V) was set to a value at which salicylate cannot be detected (full details are given in the legend to Fig. 1). 2,5-Dihydroxybenzoate was found to be eluted very close to the internal standard 3,4-dihydroxybenzoate (relative retention time 0.975), but 2,3-dihydroxybenzoate is clearly separated (Fig. 1*a*). Ether extracts of plasma samples from volunteers not taking aspirin showed no interfering peaks at this position (Fig. 1*b*). Plasma from healthy volunteers taking aspirin showed a peak at this position (Fig. 1*c* shows a typical example). However, in the small number of samples of plasma and synovial fluid from rheumatoid patients that have been studied to date, peaks of greater intensity were generally observed (e.g. Fig. 1*d*).

The retention time of the putative 2,3-dihydroxybenzoate peak in biological fluid samples relative to the 3,4-dihydroxybenzoate standard was 0.849 ± 0.008 (mean $\pm$S.D., $n = 6$), exactly the same as the value obtained in standard runs (Figs. 1*a*). By this assay method, plasma from patient A contained 230 nM-2,3-dihydroxybenzoate, and knee-joint synovial fluid from the same patient contained 240 nM-2,3-dihydroxybenzoate. These values varied by less than 5% in different runs. The 2,3-dihydroxybenzoate was quantified on the basis of standard plots of the ratio of its peak height to that of the internal standard 3,4-dihydroxybenzoate against 2,3-dihydroxybenzoate concentration at fixed 3,4-dihydroxybenzoate concentration. Although gentisate has a retention time very close to the internal standard (see the text), it was not found to alter significantly the peak height of the internal standard. The response of the electrochemical detector was directly proportional to 2,3-dihydroxybenzoate concentration in the range 0–20 μM.

In view of the large number of ether-soluble molecules present in biological fluids, an identity of relative retention times with those of a standard is inadequate evidence to attribute the peak to 2,3-dihydroxybenzoate. Two methods were used to confirm its identity. Firstly, the oxidation potential of the detector was varied in the range 0.30–0.68 V. Fig. 2 shows that the height of the putative 2,3-dihydroxybenzoate peak (expressed relative to its height at 0.68 V) varied in exactly the same way as that for a 2,3-dihydroxybenzoate standard. Other phenolic compounds give different oxidation potential curves (results not shown). Secondly, the eluent composition was varied by including methanol at concentrations up to 6% (v/v), producing large changes in retention times. However, the putative 2,3-dihydroxybenzoate peak in synovial-fluid and plasma samples gave retention times relative to the internal standard that were always identical with those given by an authentic sample of 2,3-dihydroxybenzoate.

Hence we conclude that method A provides an accurate measurement of 2,3-dihydroxybenzoate concentrations in human plasma and synovial fluid, provided that the identity of the peaks is always confirmed by the above procedures.

Method B

The eluent was changed to include 2.8% (v/v) methanol and a higher oxidation potential was used for the detector, so that salicylate could be measured (for full details see the legend to Fig. 3). The main difference was that ethyl acetate was used to extract the biological fluids, instead of diethyl ether. Unlike diethyl ether, ethyl acetate is allegedly specific for the extraction of phenolic acids [20]. However, we found this not to be absolutely true. The extraction efficiency of compounds was determined by adding them to plasma samples from volunteers who had not taken aspirin and carrying them through the

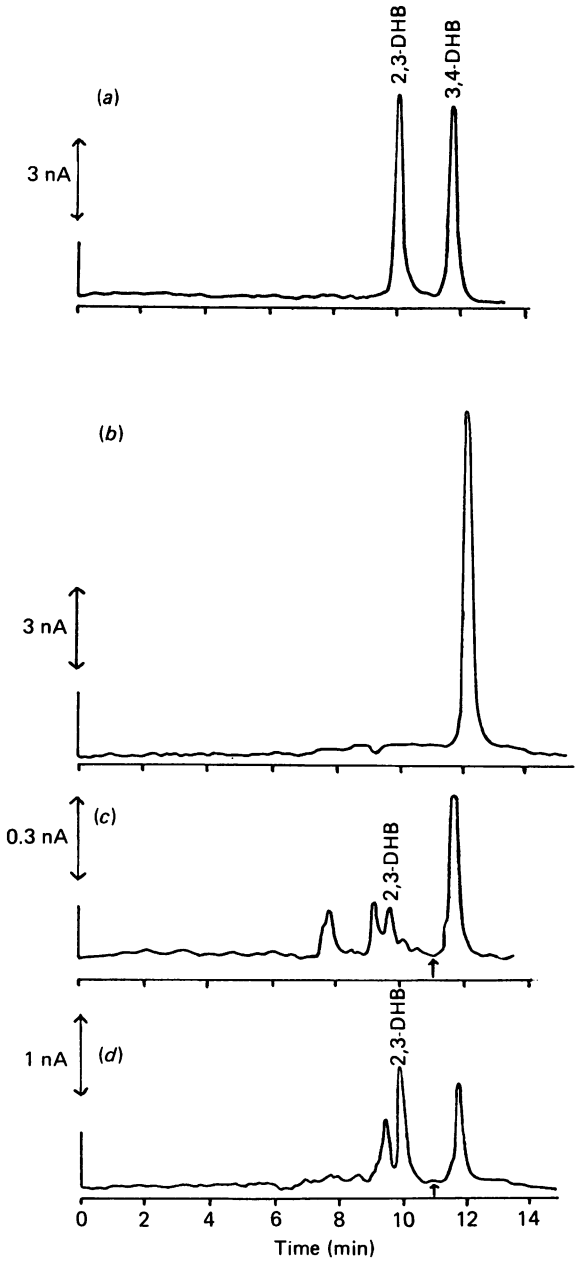


Fig. 1. Identification of 2,3-dihydroxybenzoate in human body fluids by method A

The eluent was 100% 30 mM-sodium citrate/27.7 mM-acetate buffer (pH 4.75), without methanol, at a flow rate of 1.0 ml/min. The temperature was ambient. Samples (1.0 ml) of human plasma or synovial fluid were treated with 20 μl of 0.5 mM-3,4-dihydroxybenzoate (internal standard) and 50 μl of 1 M-HCl and extracted with 10 ml of h.p.l.c.-grade diethyl ether on a vortex mixer for 2 min. After separation the ether layer was evaporated in a water bath at 40 °C and the residue was dissolved in 200 μl of mobile phase plus 50 μl of 1 M-HCl. Samples not analysed immediately were stored at −20 °C until used (this storage procedure did not affect the results). (*a*) Separation of a standard mixture of 2 μM each of 2,3-dihydroxybenzoate and 3,4-dihydroxybenzoate. Maximum sensitivity was 30 nA, detector potential +0.60 V. (*b*) Separation of an extract from a plasma sample from a healthy control subject not consuming aspirin (+0.68 V). (*c*) Separation of a plasma extract from a control subject consuming aspirin (+0.60 V). (*d*) Separation of a knee-joint synovial-fluid extract from a rheumatoid patient (patient A) consuming aspirin (+0.68 V). Arrows indicate a change in the electrochemical-detector sensitivity. Abbreviations: 2,3-DHB, 2,3-dihydroxybenzoate; 3,4-DHB, 3,4-dihydroxybenzoate.

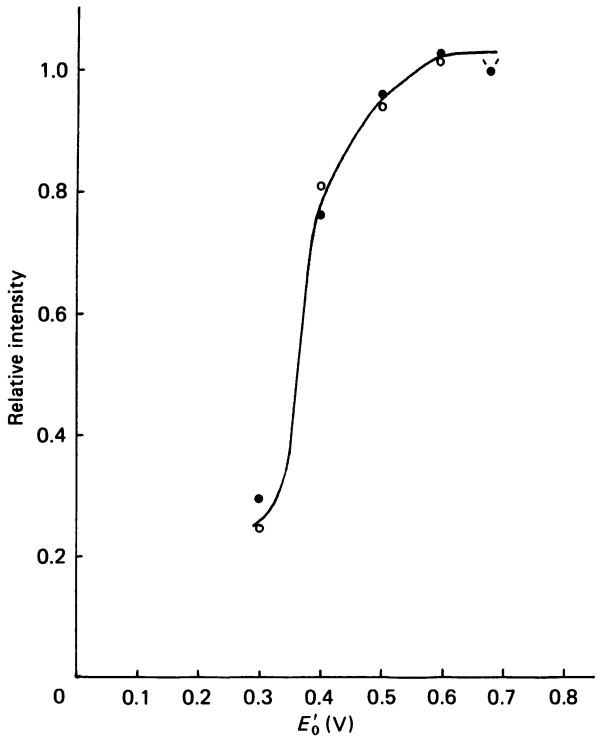


Fig. 2. Electrochemical evidence for the presence of 2,3-dihydroxybenzoate in human body fluids

The oxidation potential of the detector was varied and the height of the putative 2,3-dihydroxybenzoate peak in rheumatoid synovial fluid was measured. The relative peak height is plotted as a function of oxidation potential (●). Similar experiments were conducted with a 2,3-dihydroxybenzoate standard (○) and it may be seen that the two curves match closely.

extraction procedure. None of the compounds was present in measurable amount in the 'blank' serum sample (Fig. 3*b*). The extraction efficiency of 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate was 76±2%, that of salicylate 82±2% and that of salicylurate 81±3% (all values means±S.D., $n = 3$). Resorcinol, which is not a phenolic acid, was also found to be extracted. Resorcinol (extraction efficiency 44±3%; mean±S.D., $n = 3$) was used for the calculation of relative retention times. Method B produced an effective separation of 2,3-dihydroxybenzoate (retention time relative to resorcinol 0.434–0.442) and 2,5-dihydroxybenzoate (relative retention time 0.477–0.481), but salicylate (relative retention time 1.319–1.325) and catechol (relative retention time 1.304) ran very close to each other. Fig. 3(*a*) shows a chromatogram of a standard sample containing 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and resorcinol.

Fig. 3(*c*) shows a chromatogram of an ethyl acetate extract of plasma from a volunteer taking aspirin. Small amounts of 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate were present. Rheumatoid plasma or synovial-

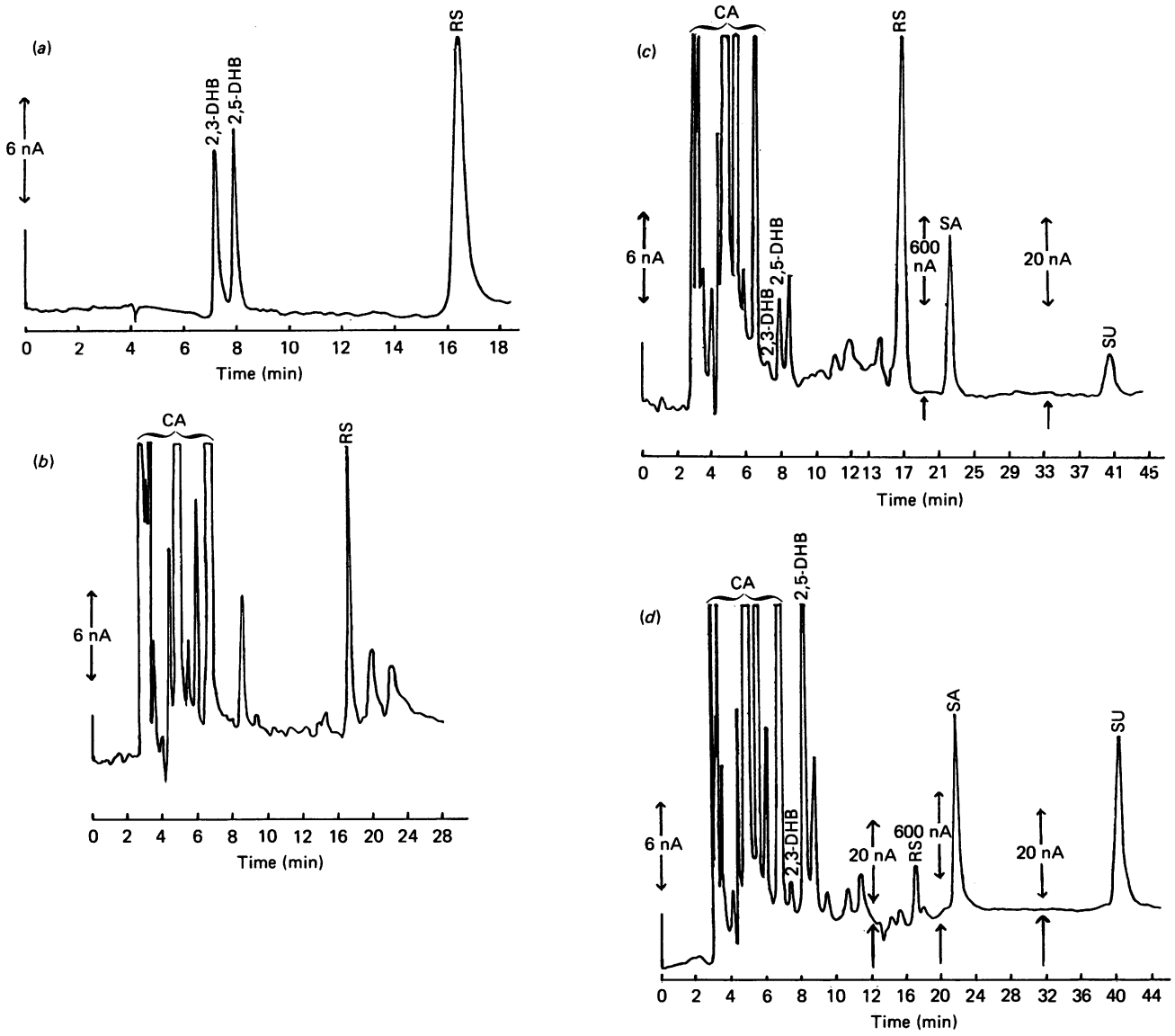


Fig. 3. Identification of salicylate-derived products in human body fluids by method B

The eluent was 97.2% (v/v) 30 mM-sodium citrate/27.7 mM-acetate buffer (pH 4.75)/2.8% (v/v) methanol at a flow rate of 0.9 ml/min. Temperature was ambient. Samples (0.20–1.0 ml) of human plasma or synovial fluid were treated with a standard solution of resorcinol (final concentration 1.0 μM) and 50 μl of 1 M-HCl. Samples were extracted with two 8 ml portions of h.p.l.c.-grade ethyl acetate on a vortex mixer for 2 min. The ethyl acetate layer was evaporated to dryness in a water bath at 55 °C, and the residue was dissolved in 200 μl of h.p.l.c.-grade water and 50 μl of 1 M-HCl. Samples not analysed immediately were stored at −20 °C until used (this storage procedure did not affect the results). (*a*) Separation of a standard mixture of 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and resorcinol (1 μM each). (*b*) Separation of an extract of a plasma sample from a healthy volunteer not consuming aspirin. (*c*) Separation of an extract from plasma of a healthy volunteer taking aspirin. The large peaks to the left of the chromatogram (marked CA) are probably catecholamines, which are efficiently extracted by ethyl acetate, but not by ether (Fig. 1). (*d*) Separation of an extract from rheumatoid plasma (patient A). Large catecholamine peaks (CA) are again present, but do not interfere with the peaks of interest. Arrows indicate a change in the electrochemical-detector sensitivity. Note the change in the time scale at 12 min (*b* and *d*) and 13 min (*c*). Detector potentials were all +0.96 V. Abbreviations: 2,3-DHB, 2,3-dihydroxybenzoate; 2,5-DHB; 2,5-dihydroxybenzoate; RS, resorcinol; SA, salicylate; SU, salicylurate.

fluid extracts generally showed greater amounts of these products in the small number of samples studied to date (Fig. 3*d* shows a typical chromatogram for rheumatoid plasma). Identification of the peaks in Figs. 3(*c*) and 3(*d*) as 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate was based on three pieces of evidence. Firstly, their retention times relative to resorcinol were identical with those of authentic samples of these compounds. Secondly, these relative retention times remained the same when the mobile phase was varied by including different amounts of methanol, even though the actual retention times of all the compounds varied markedly. Thirdly, ratios of peak height responses at different detector oxidation potentials (in the range 0.40–0.96 V)

Table 1. Concentrations of salicylate and its derivatives in body fluids, determined by chromatographic method B

Fluid taken	Concentration			
	2,5-Dihydroxybenzoate (nM)	2,3-Dihydroxybenzoate (nM)	Salicylate (μM)	Salicylurate (μM)
Plasma, patient A	1610	235	236	30.1
Synovial fluid, patient A	1710	240	192	23.2
Plasma, patient B*	596	97	186	8.1
Plasma, patient C*	620	92	206	13.4
Plasma, control on same aspirin dose as patients B and C*	247	55	196	6.6
Plasma, control on twice the aspirin dose as patients B and C*	498	52	367	5.7

* All fluid samples taken at 4.5 h after aspirin consumption. Results in different chromatographic runs varied by less than 5%. The amount of catechol that might overlap into the salicylate peak is negligible in relation to the amount of salicylate present (see under 'Method C' in the Results section). Chromatographic peaks were quantified on the basis of peak heights and comparison with the appropriate standard mixture.

were identical with those shown by the authentic compounds (Fig. 2 illustrates the principle of this method).

Table 1 shows some typical analytical results obtained by applying method B to human body fluids. The values obtained for 2,3-dihydroxybenzoate were, in a series of runs, within 5% of these given by method A. The results in Table 1 emphasize the point that the product of interest in this study (2,3-dihydroxybenzoate) is present in concentrations about two orders of magnitude less than the 'major' metabolites of salicylate, such as salicylurate.

Method C

By changing the eluent to 96% citrate/acetate buffer (pH 4.75)/4% (v/v) methanol, it was found possible to separate catechol and salicylate (retention times on a typical chromatogram were 11.8 min for catechol and 13.4 min for salicylate at an eluent flow rate of 1.0 ml/min and ambient temperature). Diethyl ether extracts of rheumatoid synovial fluid (patient A) were analysed on this system. It was necessary to extract 3.4 ml of synovial fluid in order to obtain a measurable peak in the region at which catechol would be expected to run. We were unable to obtain clear evidence that this peak was in fact catechol. However, these studies did show that the amount of catechol apparently present was insignificant in relation to the amount of salicylate (see the legend to Table 1).

DISCUSSION

Attack by 'OH radicals, generated by a Fenton system at pH 7.4, upon salicylate produces three products (Scheme 1). Formation of the decarboxylation product catechol has not been reported in previous experiments in which salicylate was exposed to Fenton systems under physiological conditions [9, 16], but its formation is not unexpected, since radiolysis of aerated salicylate solutions produces some catechol [17]. Also, decarboxylation of benzoate has been used as assay for 'OH radicals *in vitro* [18]

Salicylate reacts with 'OH with a rate constant of about 5×10^9–10^{10} $M^{-1} \cdot s^{-1}$ [21]. The fairly low oral doses of aspirin used in our studies gave body-fluid salicylate concentrations of up to 0.4 mM, which might feasibly intercept some 'OH radicals, to an extent depending on the concentrations, and reaction rate constants, for other molecules present that react with 'OH radicals. If oral aspirin is increased to about 4.2 g daily, fluid salicylate concentrations rise to over 0.8 mM [22]. The limited studies performed to date suggest that salicylate, and products derived from it, are present in the synovial fluids at concentrations similar to those in plasma.

The purpose of the present paper is to describe chromatographic methods for the specific identification and quantification in body fluids of products of 'OH attack on salicylate, in the hope that the methodology may be of use to scientists attempting to detect and measure 'OH generation in other examples of oxidative stress. 2,3-Dihydroxybenzoate can be accurately measured by the techniques described here, whereas catechol, which is formed in much smaller amounts by 'OH attack (Scheme 1), seems a less promising product to measure (see under 'Method C' in the Results section).

We stress that the limited studies on human samples reported in the present paper in no way prove that the 2,3-dihydroxybenzoate detected in body fluids does originate from radical attack on salicylate. Indeed, the presence of low concentrations of this product in the plasma of healthy human volunteers after aspirin ingestion might be taken to suggest that it can be generated by a previously unreported minor metabolic pathway. It might also be related to the 'baseline' rate of intracellular 'OH formation from ionizing radiation and Fenton reactions [2]. Further work to investigate the origin of 2,3-dihydroxybenzoate is therefore required. It should also be noted that the principle behind our methodology can be applied to other aromatic compounds.

We are grateful to Dr. David Blake for the provision of fluid samples from rheumatoid patients, and to the Arthritis and Rheumatism Council, the Wellcome Trust and CIBA–Geigy Pharmaceuticals for financial support. B.H. is a Lister Institute Research Fellow.

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Received 13 December 1985/4 February 1986; accepted 24 March 1986