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2,3-DIHYDROXYBENZOIC ACID IS A PRODUCT OF HUMAN ASPIRIN METABOLISM

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Abstract—2,3-Dihydroxybenzoic acid is present in blood plasma and urine of healthy human volunteers after aspirin ingestion. Its identity has been confirmed by mass spectrometry and by electrochemical analysis. Methods for its identification and measurement are described. The concentration of 2,3-dihydroxybenzoic acid is much lower than that of 2,5-dihydroxybenzoic acid, salicylic acid or salicyluric acid.

Aspirin (*O*-acetylsalicylic acid) is a widely-used analgesic for self-medication, and larger doses are sometimes used in the treatment of osteoarthritis and rheumatoid arthritis. It has also been suggested as a prophylactic agent against thromboembolic vascular disease. Aspirin is quickly hydrolysed to salicylic acid (2-hydroxybenzoic acid) in the body. Some salicylic acid is excreted as such, some is conjugated with glycine to produce salicyluric acid, some is hydroxylated to gentisic acid (2,5-dihydroxybenzoic acid) and some is conjugated with glucuronic acid (Fig. 1) [1–3]. The therapeutic action of aspirin may be

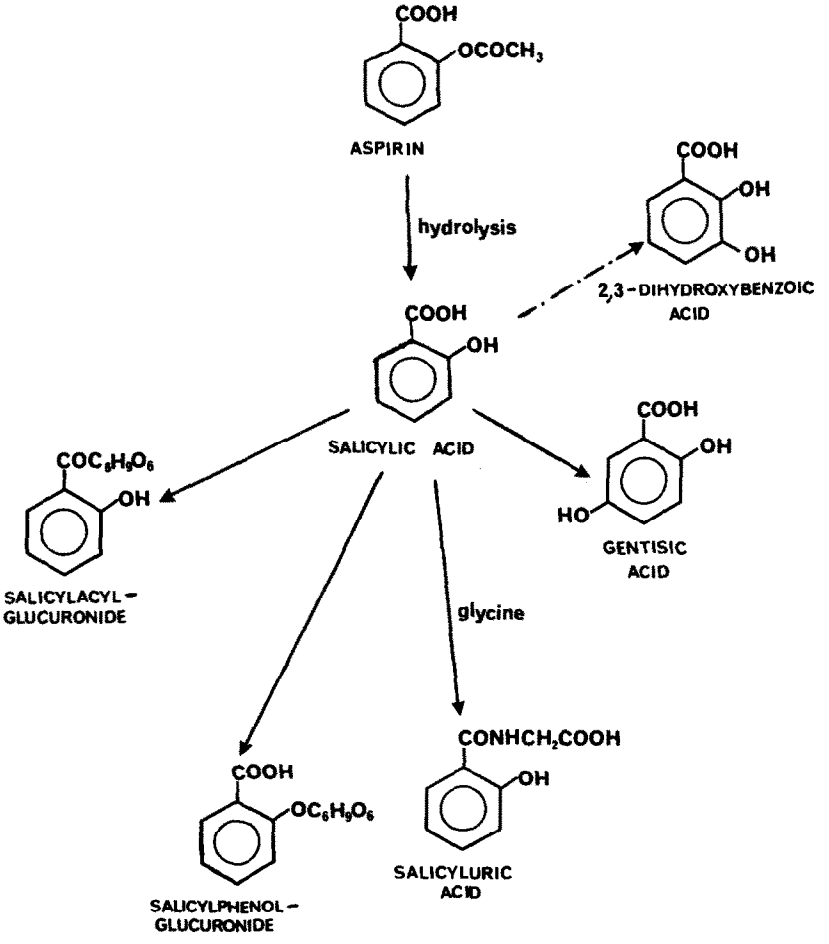
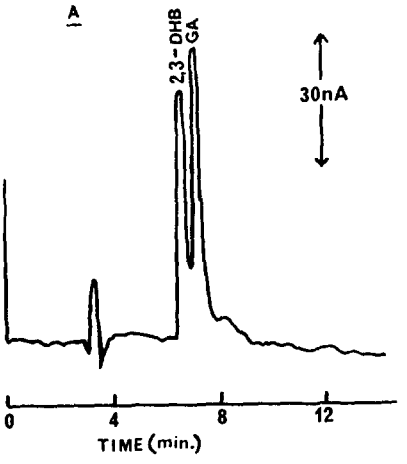
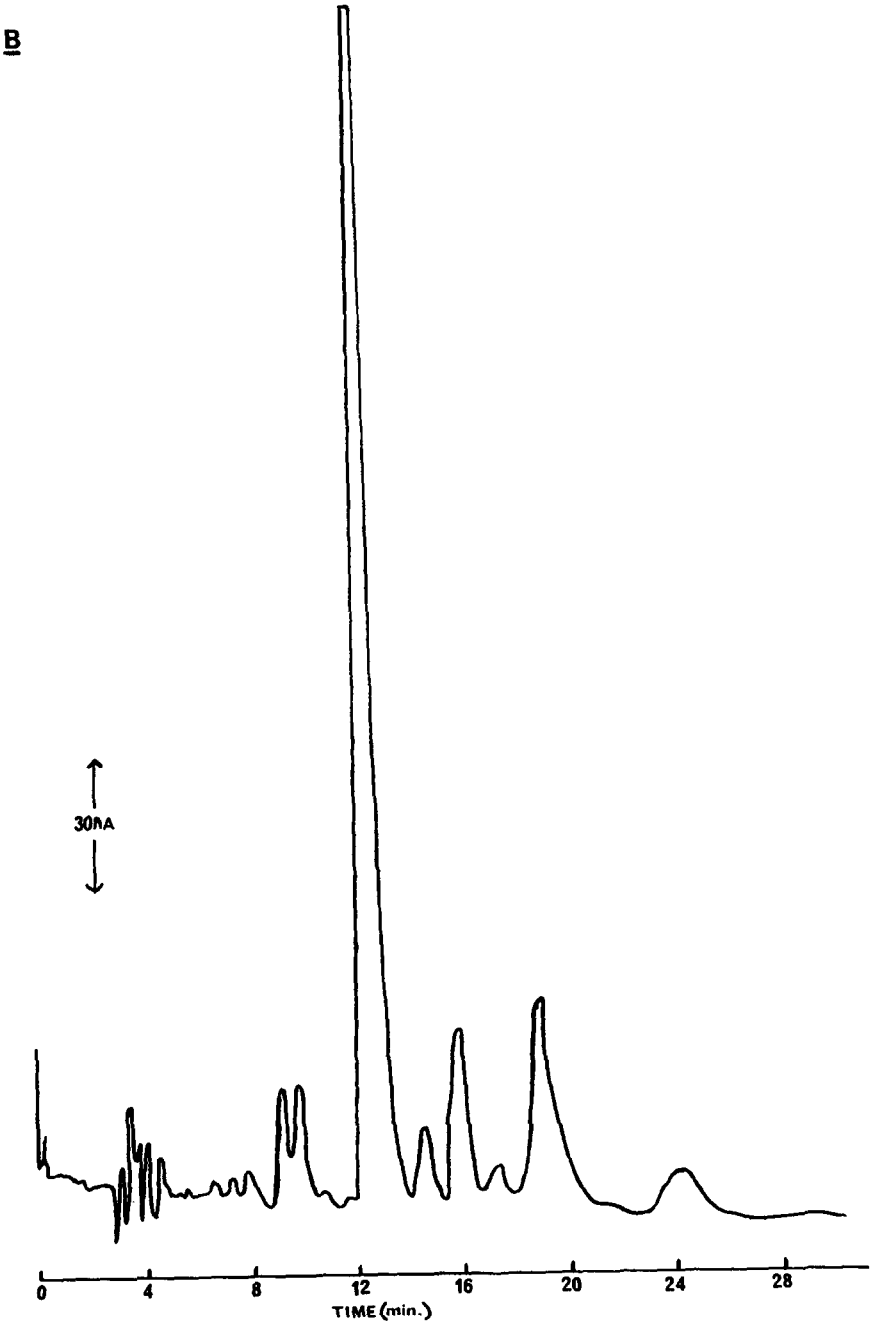


Fig. 1. Major reported metabolites of salicylic acid.


A
2,3-DHB
GA
30nA
0
4
8
12
TIME (min.)


B
30nA
0
4
8
12
16
20
24
28
TIME (min.)

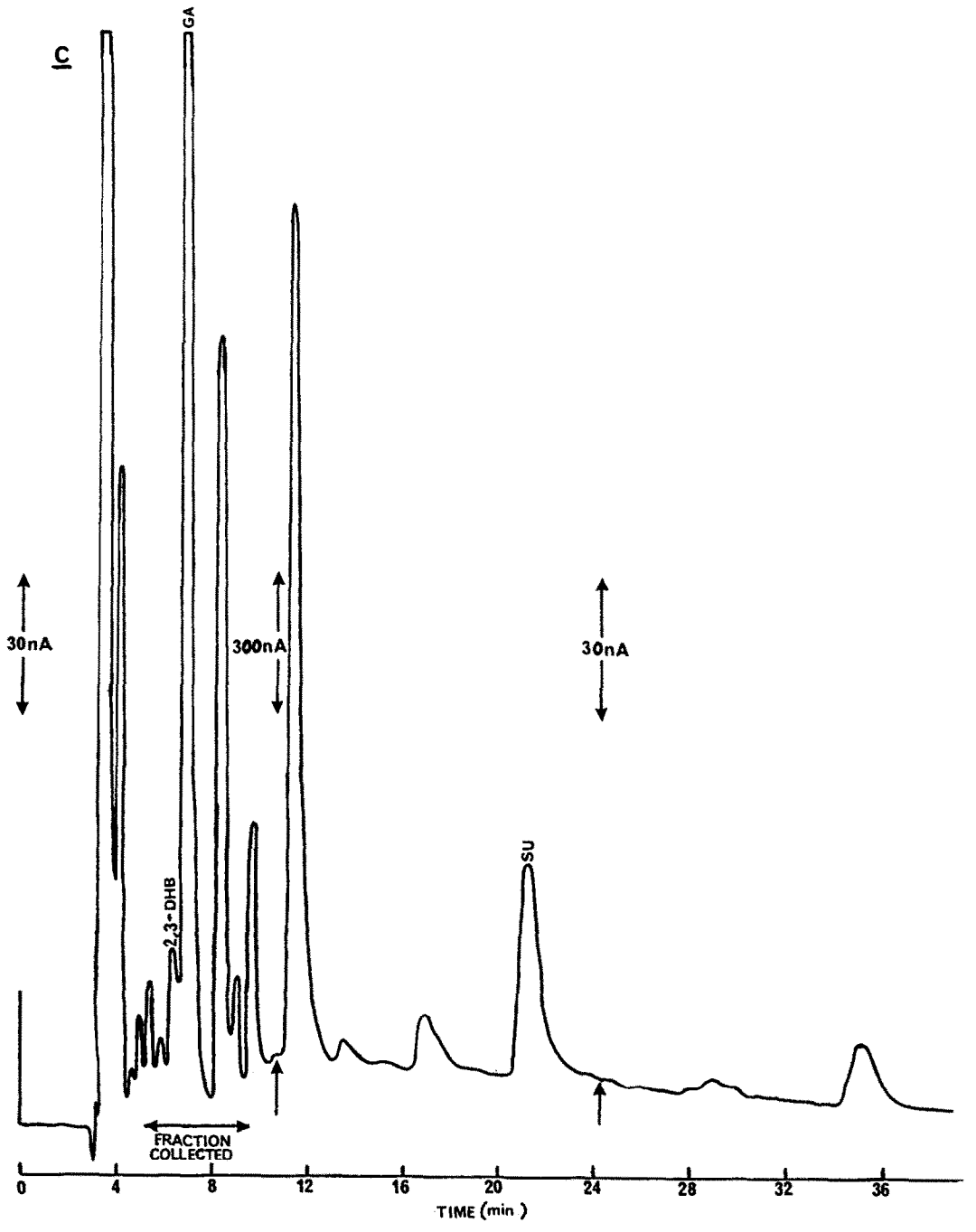


Fig. 2. HPLC analysis of aspirin metabolites in human urine. (A) Separation of a standard mixture of 6 μM each of 2,3-dihydroxybenzoic acid and gentisic acid. Mobile phase I was used (for composition see the Materials and Methods section). Electrochemical detector potential + 0.76V. (B) HPLC profile of a diethyl ether extract of urine (after 1 in 50 dilution) from a healthy adult male volunteer not consuming aspirin. (C) HPLC profile of a diethyl ether extract of urine (after 1 in 20 dilution) from a healthy adult male volunteer, voided 3 hr after ingesting 1200 mg of aspirin B.P. Arrows indicate a change in the electrochemical detector sensitivity. Abbreviations used: 2,3-DHB, 2,3-dihydroxybenzoic acid; GA, gentisic acid; SU, salicyluric acid.

related not only to its ability to inhibit cyclooxygenase, but possibly also to its effects on neutrophil activation [4] and leukotriene production [5]. Aspirin and salicylic acid may act additionally as scavengers of reactive oxygen radicals, such as hydroxyl (·OH) [6]; these are thought to be important in aggravating the inflammatory process [7, 8].

The compound 2,3-dihydroxybenzoic acid (Fig. 1) has been evaluated as a chelating agent for the prevention of transfusion-related iron overload in thalassaemic patients [9]. Binding of iron salts to this compound appears to diminish their reactivity in promoting lipid peroxidation and iron-dependent formation of ·OH radicals from H_2O_2 [10, 11]. Indeed, 2,3-dihydroxybenzoic acid has been reported to decrease acute lung injury mediated by neutrophil activation and consequent free radical production [11]. However, 2,3-dihydroxybenzoic acid has not been reported to date as a product of human aspirin metabolism [1–3, 12, 13]. In the present paper we report studies showing the presence of this product in plasma and urine from healthy adults consuming aspirin. 2,3-Dihydroxybenzoic acid was separated from other aspirin metabolites by high-performance liquid chromatography (HPLC), and was quantitated electrochemically. Its identity was confirmed by electrochemistry and by gas chromatography–mass spectrometry.

MATERIALS AND METHODS

Reagents. HPLC-grade solvents were obtained from BDH Chemicals Ltd and aromatic compounds from Aldrich. Standard solutions were made up and stored as described in [15].

HPLC. HPLC was carried out on an HPLC Technology Ltd or a Jones Chromatography Ltd Spherisorb 5 ODS reverse-phase column (25 cm × 4.6 mm) using an EDT LCA15 electrochemical detector equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode. The mobile phases (composition described below) were sparged continuously with helium gas during elution. The injection loop was cleaned at least 3 times with 100 μl HPLC-grade water after each injection.

Urine sample analysis by HPLC. Samples of freshly-voided human urine (5.0 or 10.0 ml) were acidified with 1.0 ml of 1 M HCl, and then extracted twice with 10 ml portions of HPLC-grade diethyl ether (ethoxyethane). The combined diethyl ether extracts were evaporated to dryness in a water-bath at 40° and then reconstituted with 250 μl of 0.20 M HCl. The reconstituted samples were diluted 1 in 20 or 1 in 50 with further 0.20 M HCl and then frozen until analysis. The eluent was 90% (v/v) sodium citrate (30 mM)–acetate (27.7 mM) buffer pH 4.75 and 10% (v/v) methanol (mobile-phase I) at a flow-rate of 1.0 ml/min. For the rechromatography experiment described in the text, the eluent was sodium citrate (30 mM)–acetate (27.7 mM) buffer pH 4.75 (mobile-phase II), at the same flow rate.

Plasma preparation and analysis. Blood samples were drawn from healthy adult volunteers (who had given informed consent) into heparinized tubes and centrifuged immediately. The supernatant (1.0 ml) was treated with 10 μl of a 1.00 mM solution of 3-hydroxybenzoic acid (internal standard) plus 50 μl of 1 M HCl, and then extracted with 10 ml of HPLC grade diethyl ether. Extracts were evaporated to dryness and reconstituted in the same way as for the urine extracts.

Gas chromatography–mass spectrometry

The urine extract and reference standards of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid (approximately 50 μg of each) were flash methylated with ethereal diazomethane, followed by immediate removal under nitrogen of excess reagent.

GC–MS analyses were carried out using a VG7070E mass spectrometer linked to a Hewlett Packard 5790A gas chromatograph and a VG 11-250 data system. Separations were performed on a 25 m BP10 capillary column (S.G.E.) operated at a temperature of 190° with helium as carrier gas. The injector temperature was 220°, and samples (1 μl) were injected with a split ratio of 30:1. Electron impact mass spectra were obtained at an ionising voltage of 20 eV with source temperature 200°.

RESULTS

Detection of 2,3-dihydroxybenzoic acid in human urine

Figure 2A shows that the HPLC technique developed achieved a clear separation of 2,3-dihydroxybenzoic acid from 2,5-dihydroxybenzoic acid (gentisic acid). Ether extracts of urine samples from healthy adult volunteers showed no interfering peaks at these positions (Fig. 2B shows a typical example). Figure 2C shows a typical chromatogram of an ether extract of urine from an adult male volunteer 3 hr after ingesting 1.2 g of aspirin (B.P.) It may be seen that peaks are present at the retention times expected for 2,3-dihydroxybenzoic acid (6.62 min), gentisic acid (7.10 min) and salicyluric acid (21.44 min). Salicylic acid co-elutes with a peak present in normal human urine and thus could not be quantitated in these experiments.

In view of the large number of ether-soluble molecules likely to be present in human urine, a retention time identical to a standard is insufficient evidence to attribute a peak to 2,3-dihydroxybenzoic acid. The identity of the putative 2,3-dihydroxybenzoic acid peak was confirmed in three ways. Firstly, its electrochemical behaviour was studied. The oxidation potential of the electrochemical detector was altered in the range of 0.25–0.62 V. The peak height ratios at different potentials corresponded almost exactly to those obtained using authentic 2,3-dihydroxybenzoic acid (Table 1). Other aromatic compounds, such as gentisic acid, showed markedly different electrochemical behaviour (data not shown). Secondly, 20 μl samples of the initial urine extract were chromatographed using mobile phase I as in Fig. 2C and a fraction covering the retention time range 5.0–9.6 min was collected (the electrochemical detector was switched off to prevent oxidation of the eluting compounds). This fraction was evaporated to dryness, the residue dissolved in 0.8 ml 0.2 M HCl and the resulting solution rechromatographed using a different mobile phase

Table 1. Identification of the putative 2,3-dihydroxybenzoic acid peak in an extract of human urine by comparison of peak height ratios with those obtained using a 2,3-dihydroxybenzoic acid standard

Peak height ratio	Value for sample	Value for pure 2,3-dihydroxybenzoic acid
0.55V/0.62V	0.9272	0.9613
0.45V/0.62V	0.7739	0.8048
0.35V/0.62V	0.4828	0.4471
0.25V/0.62V	0.1648	0.1714
0.45V/0.55V	0.8347	0.8372
0.35V/0.55V	0.5207	0.4651
0.25V/0.55V	0.1777	0.1783
0.35V/0.45V	0.6238	0.5556
0.25V/0.45V	0.2129	0.2130
0.25V/0.35V	0.3413	0.3833

Chromatography was performed using mobile phase I as described in the Materials and Methods section.

(mobile phase II, described in the Materials and Methods section). The retention times of the peaks (2,3-dihydroxybenzoic acid 10.86 min; gentisic acid 14.24 min) had changed (Fig. 3B) in exactly the same way as those of the authentic compounds (Fig. 3A).

Thirdly, the urine extract, and standards of authentic 2,3- and 2,5-dihydroxybenzoic acids, were flash methylated with diazomethane and subjected to capillary gas chromatography–mass spectrometry. Data were obtained in the total ion current scanning mode. The gas chromatograph achieved a clear separation of the methyl esters of 2,3- and 2,5-dihydroxybenzoates from other isomers, as may be seen from the relative retention times listed in Table 2. Figure 4 show the reconstructed total ion current (TIC) chromatogram and the mass chromatogram of m/z 168 for the methyl esters of a reference mixture of 2,3- and 2,5-dihydroxybenzoic acids. Mass chromatograms for m/z 168 and m/z 136 ions from the urine extract show similar profiles (Fig. 5), with a significant peak corresponding to the methyl ester of 2,5-dihydroxybenzoic acid and a smaller peak for the methyl ester of 2,3-dihydroxybenzoic acid. In addition to having retention times the same as the reference standards (Table 2), the mass spectra are also comparable. For example, Fig. 6 shows a comparison of the electron impact mass spectra of the methyl esters of genuine 2,5-dihydroxybenzoic acid and of the corresponding component from the urine extract. A similar comparison for 2,3-dihydroxybenzoic acid also produced matching mass spectra (not shown).

Further evidence for the presence of 2,3-dihydroxybenzoic acid in urine was obtained by considering dimethylated products. The mass chromatogram of m/z 182 showed three components (Fig. 7), the identities of which were assigned by the spectra from individual reference standards. Figure 8 shows one example; it may be seen that the mass spectra match very closely.

Table 2. Relative retention times on gas chromatography of the methyl esters of dihydroxybenzoic acid isomers

Isomer	Relative retention time
2,3	1.00
2,6	1.31
2,5	2.72
2,4	3.34
3,4	8.76

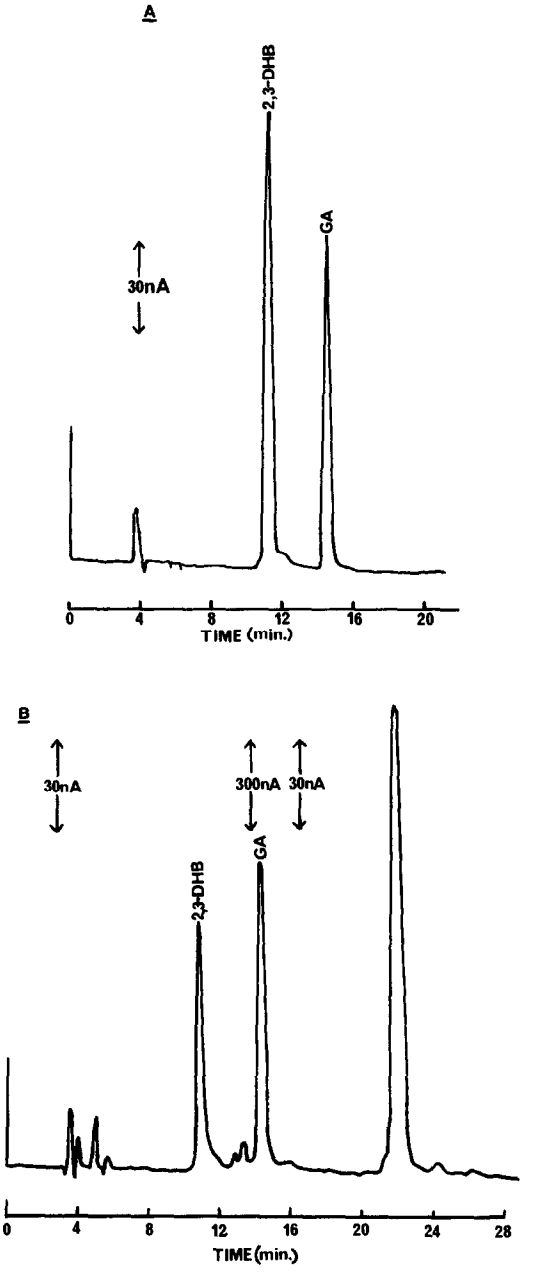


Fig. 3. Rechromatography of aspirin metabolites. A fraction covering the retention times 5.0–9.6 was collected as shown in Fig. 2C, except that the electrochemical detector was switched off. The fraction was evaporated to dryness, the residue dissolved in 0.8 ml of 0.2 M HCl and rechromatographed using a different mobile phase (phase II)—see the Materials and Methods section. (A) Chromatography of a standard mixture of 10 μM each of 2,3-dihydroxybenzoic acid and gentisic acid in mobile phase II. (B) Rechromatography of the urine extract. Electrochemical detector potential + 0.76V.

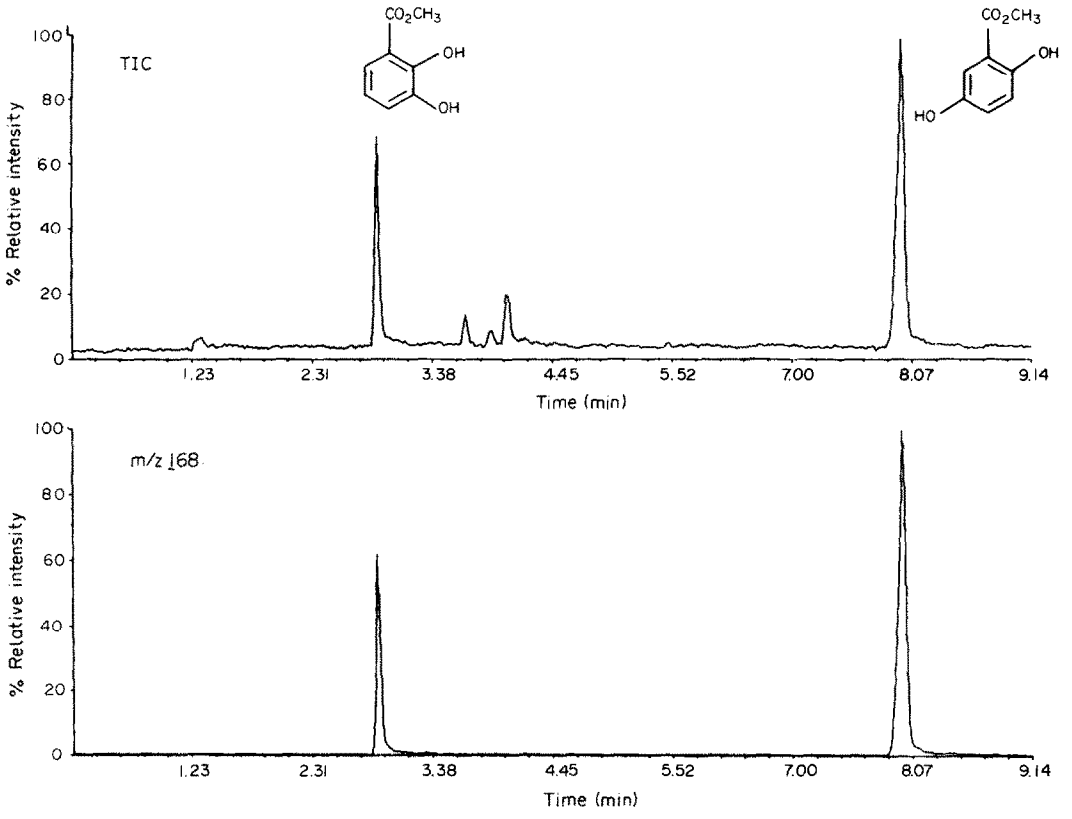


Fig. 4. Gas chromatography–mass spectrometry of the methyl esters of 2,3- and 2,5-dihydroxybenzoic acid. The upper plot is the reconstructed total ion current chromatogram and the lower plot is the mass chromatogram of m/z 168 for the methyl esters of a reference mixture of the above two compounds.

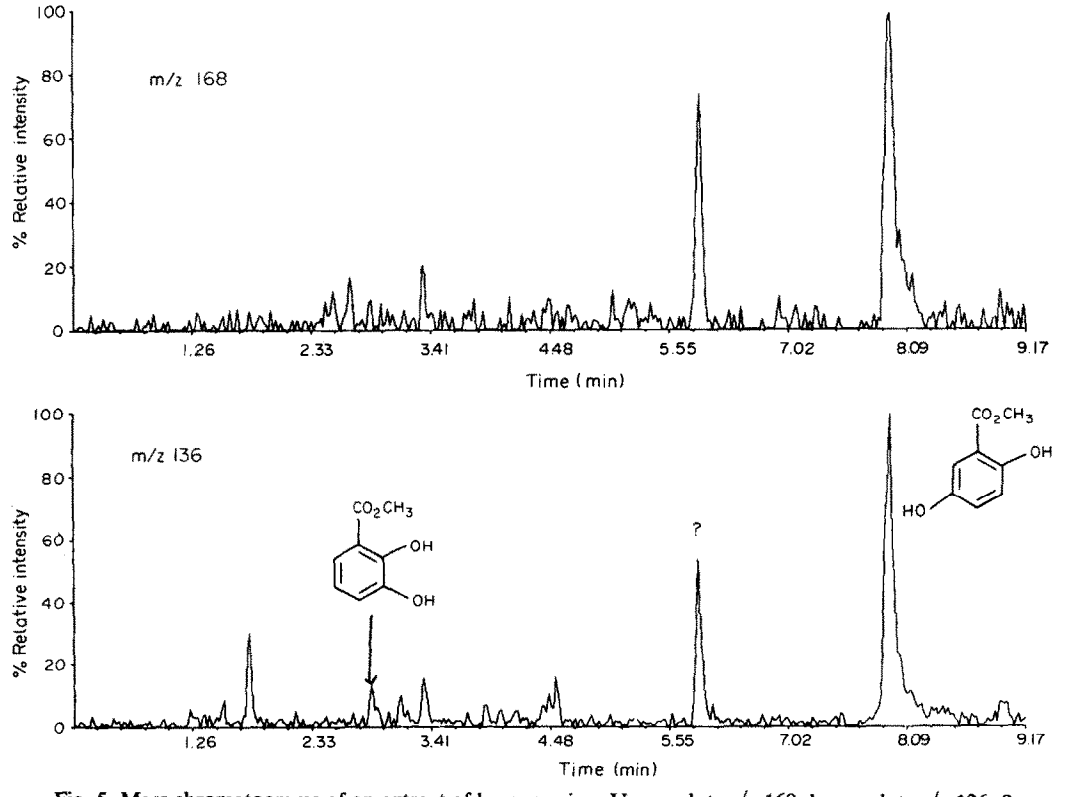


Fig. 5. Mass chromatograms of an extract of human urine. Upper plot m/z 168; lower plot m/z 136. ?: identity of component uncertain.

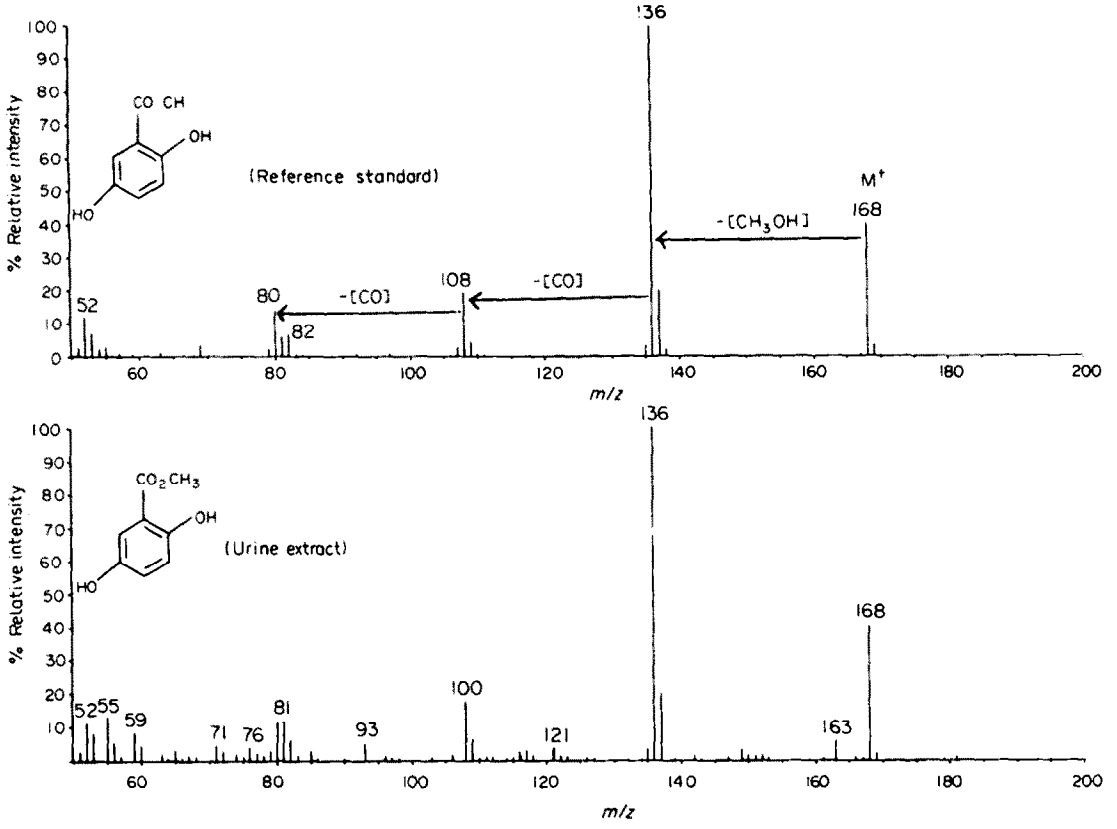


Fig. 6. Electron impact mass spectra of the methyl ester of 2,5-dihydroxybenzoic acid. Upper plot, reference standard. The peak at m/z 168 is the molecular ion of the methyl ester. The lower plot shows the mass spectrum of the putative 2,5-dihydroxybenzoic acid methyl ester peak from the urine extract.

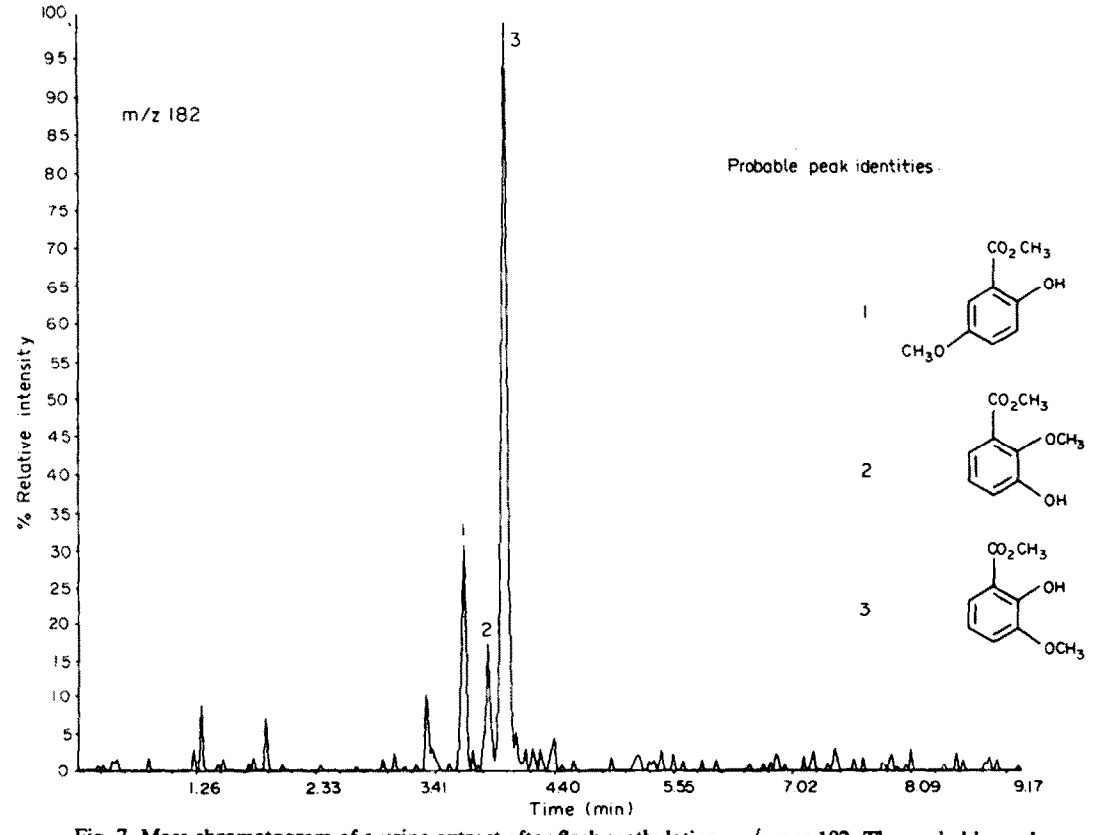


Fig. 7. Mass chromatogram of a urine extract after flash methylation. m/z was 182. The probable peak identities are shown, assigned from the spectra of reference compounds.

Table 3. Concentrations of aspirin metabolites in human body fluids

Subject	Fluid analysed		[2,3-dihydroxy-benzoic acid]	[gentisic acid]	[salicyluric acid]	[salicylic acid]
Healthy adult male, aged 28 yr	Urine, 3 hr after 1.2 g of aspirin		4.2 μM	46.8 μM	451 μM	ND*
Healthy adult male, aged 28 yr	Plasma, x hr after 1.2 g of aspirin	$x = 0$	nil	nil	nil	nil
		$x = 1$	trace	0.01 μM	2.75 μM	131 μM
		$x = 2$	0.16 μM	0.5 μM	7.76 μM	617 μM
		$x = 4.5$	0.05 μM	0.5 μM	5.65 μM	367 μM
Healthy adult male, aged 36 yr	Plasma, x hr after 0.6 g of aspirin	$x = 0$	nil	nil	nil	nil
		$x = 1$	0.03 μM	0.16 μM	5.52 μM	261 μM
		$x = 2$	0.05 μM	0.25 μM	8.46 μM	329 μM
		$x = 4.5$	0.05 μM	0.25 μM	6.59 μM	196 μM
Healthy adult female, aged 44 yr	Plasma, x hr after 0.6 g of aspirin	$x = 1$	trace	ND	4.94 μM	278 μM
		$x = 2.3$	17 nM	ND	8.76 μM	318 μM
		$x = 3.75$	16 nM	ND	7.45 μM	247 μM
		$x = 4.5$	13 nM	ND	6.37 μM	184 μM

* Not determined. Salicylic acid concentrations cannot be measured in urine by the chromatographic technique used, because of the presence of an interfering peak.

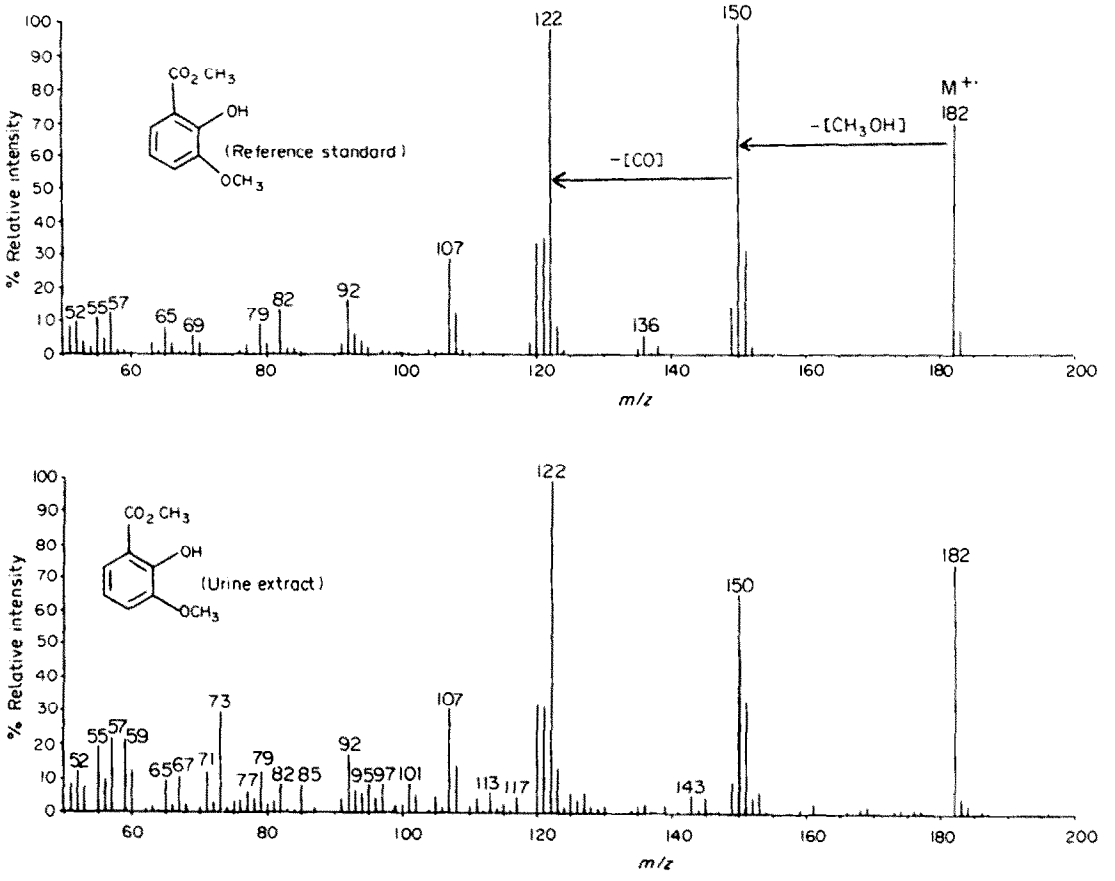


Fig. 8. Identification of a dimethylated derivative of 2,3-dihydroxybenzoic acid in human urine. The mass chromatograms of m/z 168 for a reference standard of dimethylated 2,3-dihydroxybenzoic acid and for peak 3 in Fig. 7 are shown.

2,3-Dihydroxybenzoic acid, gentisic acid and salicyluric acid were quantitated after HPLC of a urine extract by direct comparison of peak heights with those of standard mixtures extracted and subjected to HPLC under identical conditions. Table 3 shows results selected from a wide variety of measurements on different subjects. It may be seen that the concentration of 2,3-dihydroxybenzoic acid present is always much less than that of the other aspirin metabolites.

Detection of 2,3-dihydroxybenzoic acid in human blood plasma

Similar HPLC methods were used to measure the concentrations of aspirin metabolites in ether extracts of plasma samples from healthy adult volunteers after aspirin consumption. A peak corresponding to 2,3-dihydroxybenzoic acid was detected in all the samples examined and its identity established by electrochemistry as described above. Table 2 shows a selection of the results obtained. The peak plasma concentration of 2,3-dihydroxybenzoic acid was found approximately 2 hr after aspirin ingestion. Again, the concentrations of this product are considerably less than those of gentisic acid or salicyluric acid.

DISCUSSION

In the present paper we have rigorously established the presence of 2,3-dihydroxybenzoic acid in blood plasma and urine of healthy adult volunteers after consuming aspirin. This product may have escaped previous detection because its concentration is so much lower than that of other salicylic acid metabolites (Table 3). It may be that the hydroxylase enzyme producing gentisate also produces 2,3-dihydroxybenzoic acid as a minor product. Another possibility is that some 2,3-dihydroxybenzoic acid might arise as a product of attack of hydroxyl radical, generated *in vivo* [7], on the salicylic acid molecule [15]. In view of its ability to interfere with iron-dependent radical reactions, which are known to be important in arthritis [7, 8], it may be that 2,3-dihydroxybenzoic acid plays some part in the observed therapeutic actions of aspirin.

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