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# Determination of Hydroxyl Radicals Using Salicylate as a Trapping Agent by Gas Chromatography-Mass Spectrometry

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**Objectives:** To establish a sensitive method for measuring hydroxyl radical formation in biological systems using salicylate as probe.
**Methods:** Salicylate hydroxylation products and related aromatic compounds were extracted and converted to trimethylsilyl (TMS) derivatives. The derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS). Quantitation was achieved by selected-ion-recording (SIR) with benzoic acid (ring-D5) as an internal standard.
**Results:** All compounds were well separated and specifically quantitated by a GC-MS procedure. Standard curves were linear in the concentration ranges investigated (0.1–10 nmol) for all individual compounds. Recovery from human plasma was in the range of 90–102%. The detection limit was between 50 fmol–1 pmol per 1 μL injection. The within-run and between-run coefficients of variation were between 4.6–9.1%. We were able to detect the baseline levels of hydroxylation products in human fibroblasts after incubation with salicylate.
**Conclusions:** The GC-MS assay presented here can specifically identify and quantitate salicylate hydroxylation products and related aromatic compounds, which can be used as an *in vivo* marker of oxidative stress. This sensitive method has broad applications, both in the area of free radical medicine and in the pharmacological study of aspirin and its metabolites. *Copyright © 1997 The Canadian Society of Clinical Chemists*

KEY WORDS: hydroxyl radical; salicylic acid; catechol; dihydroxybenzoic acid; gas chromatography-mass spectrometry.

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Abbreviations: ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; ASP, aspirin; SA, salicylic acid; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,4-DHBA, 2,4-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; 2,6-DHBA, 2,6-dihydroxybenzoic acid; SUA, salicyluric acid; BA, benzoic acid; TMS, trimethylsilyl; SIR, selected-ion recording; RT, retention time.

## Introduction

There is increasing evidence to support the involvement of free radical reactions in several human diseases, such as atherosclerosis, cerebral and heart ischemia-reperfusion injury, cancer, rheumatoid arthritis, inflammation, diabetes, aging, and neurodegenerative conditions (1–4). The increasing interest in the role of free radicals in the pathogenesis of human disease has led to an greater need for techniques that measure free radicals and their by-products *in vivo* and, most importantly, in the clinical situation.

The category of "reactive oxygen species" (ROS) includes not only oxygen free radicals, but also non-radical oxygen derivatives that are involved in oxygen radical production. Among these, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$) are not very toxic. However, in the presence of transition metal ions, these species are converted to hydroxyl radicals ($^{\cdot}OH$) through a Fenton reaction or Haber-Weiss

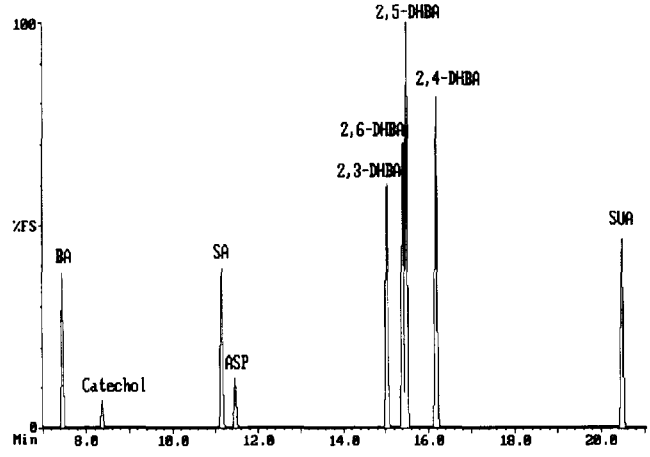
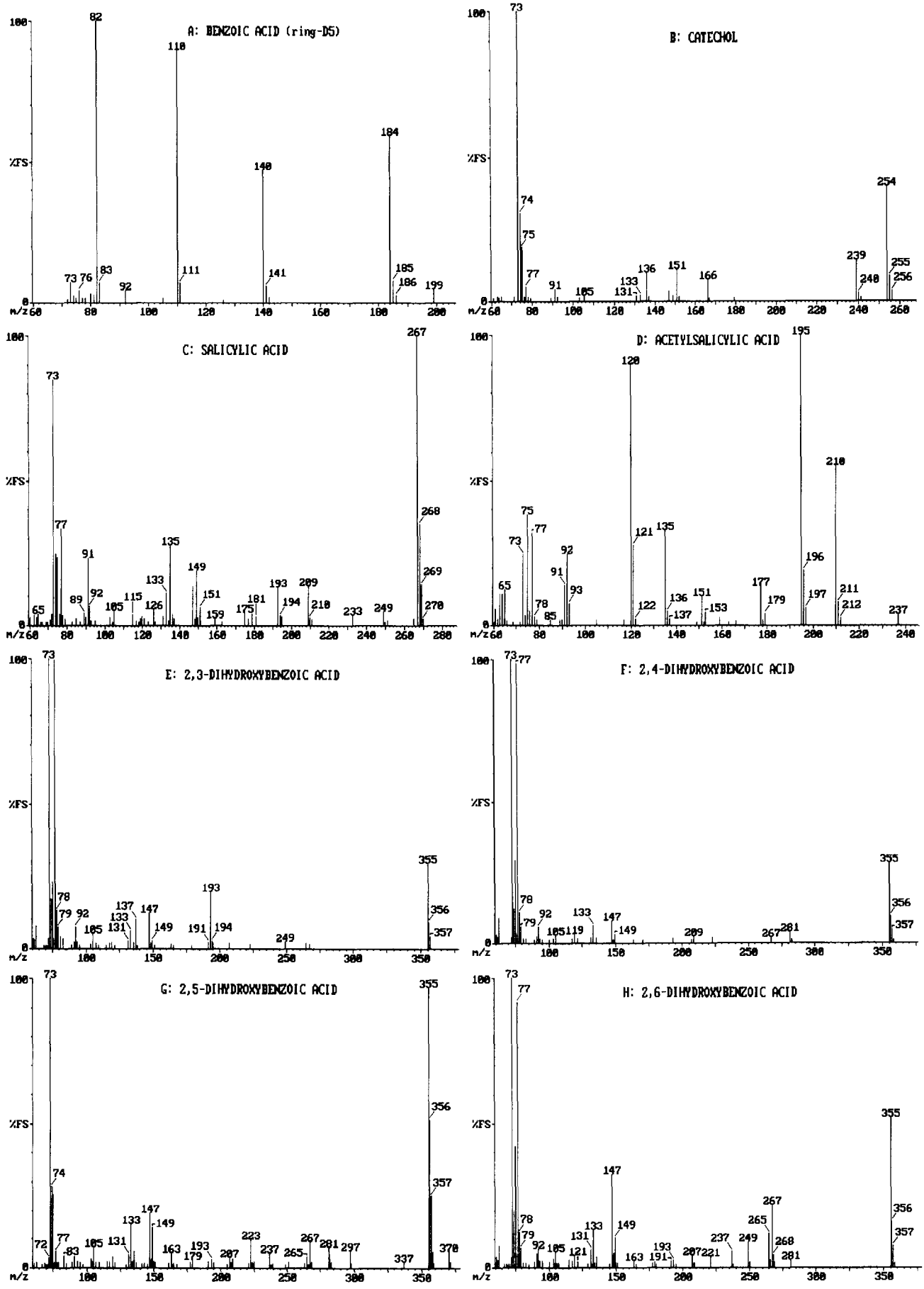



Figure 1 — GC-MS run of the whole spectrum of the TMS derivatives of salicylate hydroxylation products and related compounds in SIR mode.


A: BENZOIC ACID (ring-D5)
B: CATECHOL
C: SALICYLIC ACID
D: ACETYLSALICYLIC ACID
E: 2,3-DIHYDROXYBENZOIC ACID
F: 2,4-DIHYDROXYBENZOIC ACID
G: 2,5-DIHYDROXYBENZOIC ACID
H: 2,6-DIHYDROXYBENZOIC ACID
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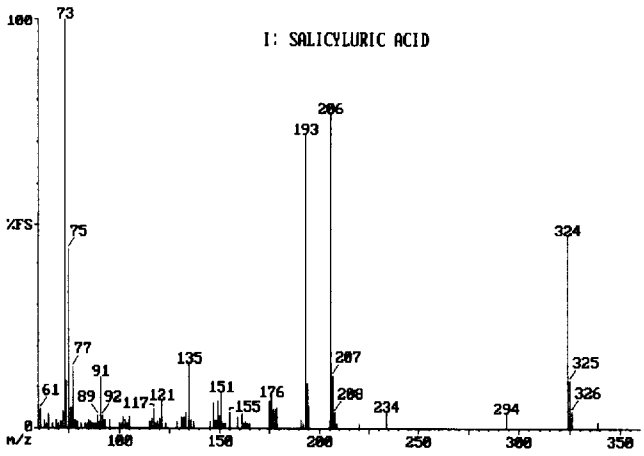


Figure 2 — Full EI mass spectra of the TMS derivatives of salicylate hydroxylation products and related compounds. A, benzoic acid (ring-D5); B, catechol; C, salicylic acid; D, acetylsalicylic acid; E, 2,3-dihydroxybenzoic acid; F, 2,4-dihydroxybenzoic acid; G, 2,5-dihydroxybenzoic acid; H, 2,6-dihydroxybenzoic acid; I, salicyluric acid.

reaction (4–6). Interaction between superoxide and nitric oxide can also form hydroxyl radicals (7). The hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion-controlled rates ($10^7$–$10^{10}M^{-1}s^{-1}$), and cause membrane lipid peroxidation, protein aggregation, and DNA hydroxylation (4). Because the half-life of hydroxyl radical in cells has been estimated to be $10^{-9}$ s, its measurement is very difficult (8). The two trapping methods with the greatest potential for specifically identifying hydroxyl radicals are electron spin resonance (ESR)-spin trapping (9) and aromatic hydroxylation (10). Although the spin-trapping method may be a definitive way to trap hydroxyl radicals, artifacts do exist and a great deal of expertise is required to interpret experimental results. The main disadvantage of all spin traps at present is that they can not detect superoxide or hydroxyl radicals *in vivo,* nor can they be administered to humans (8,11).

Aromatic compounds, such as salicylate, 3- or 4-hydroxybenzoate, aminosalicylate, tyrosine, or phenylalanine, undergo addition reactions with hydroxyl radicals, producing characteristic products of hydroxylation. These reactions have been used for measuring hydroxyl radical production by spectrophotometry or HPLC (11–14). For *in vivo* human studies, the candidate aromatic compound should be safe to administer to humans, and it should reach concentrations in body fluids sufficient to scavenge hydroxyl radicals. In addition, the hydroxylation products should be stable, be present at measurable levels even under control conditions, and their concentration should be proportional to the hydroxyl radicals formed *in vivo.* In the present study, we established a method that meets all of the above conditions to measure the hydroxylation products and related aromatic compounds by GC-MS, using salicylate as a probe.

## Materials and methods

### Chemicals

SA, ASP, catechol, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA, and SUA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BA was supplied by Cambridge Isotope Laboratories Inc. (Andover, MA, USA). N,O-Bis (trimethylsilyl) trifluroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Methanol and ethyl acetate were obtained from Baxter Co. (Mississauga, Ontario, Canada). Ethyl ether was from Fisher Scientific (Fair Lawn, NJ, USA). Sodium sulphate anhydrous was from BDN Inc. (Toronto, Ontario, Canada). All the reagents and gases used were of analytical grade. Distilled water was purified by a Milli-Q UV Plus ultrapure water system (Millipore, Molsheim, France).

### Sample extraction and derivatization

Aliquots of samples (100 μL plasma, tissue homogenate, or cell suspension) were spiked with 20 μL of 50 μM (1 nmol) BA as an internal standard. Methanol (100 μL) was added to precipitate proteins. The volume was brought to 3 mL with water, and saturated with approximately 1 g of NaCl. The pH was adjusted to 1 or less by adding 3 drops of 6 N HCl. The sample was extracted with 3 mL ethyl acetate, 3 mL ethyl ether, and again with 3 mL ethyl acetate. The organic phases were pooled, dried over sodium sulfate, and evaporated under a stream of nitrogen to dryness. BSTFA in 1% TMCS (50 μL) was added to the sample and incubated for 10 min at 60 °C to form TMS derivatives.

### Standard curves

Samples for standard curves were prepared by using increasing amounts of SA, ASP, catechol, 2,3-, 2,4-, 2,5-, 2,6-DHBA, and SUA (from 0.1 to 10 nmol of each). A fixed amount of BA (1 nmol) was added to each sample as internal standard. The samples were extracted and derivatized as described above.

### Accuracy and precision study

A standard mixture of known amounts of all the compounds listed above, plus the internal standard, was added to pure water and normal human pooled plasma. The samples were extracted and derivatized as described. The preparations were analyzed. The peak areas obtained from GC-MS were converted to concentration using appropriate standard curves, and the values from plasma and water were compared. The ratio of the concentration in the plasma to the corresponding concentration in water was used as an index of recovery. The detection limit of the assay was determined by diluting the standard mixture and determining the concentration at which the response signal in the assay was no longer sig-

nificantly different from the signal generated by a water blank. The precision of the method for the determination of catechol, 2,3-DHBA, and 2,5-DHBA was conducted on pooled samples of human plasma with a known concentration (100 nM) of these standards.

### GC-MS

The TMS derivatives were analyzed using a high-sensitivity, research grade quadrupole mass spectrometer (VG-Trio 2A) interfaced to a Hewlett Packard 5890 Series II gas chromatograph equipped with a 30 m, 0.32 μm, DB-5 capillary column. A 1-μL aliquot was injected into the GC-MS using a Hewlett Packard 7673 autosampler and a splitless injection port. The helium flow rate was 1 mL/min, the injection port and the transfer line temperature were kept at 250 °C. Column temperature was initially set at 60 °C for 0.5 min, increased by 20 °C/min until 160 °C, followed by 6 °C/min to 260 °C. Electron impact (EI) source parameters were as follows: temperature 160 °C, electron energy 70 eV, filament emission current 250 μA. A specific ion for each derivative was selected for SIR for quantitation. Response ratio of the specific ion for each compound to that of the internal standard was then converted to concentrations using the relevant standard curve.

## Results

Figure 1 shows the chromatogram of the TMS derivatives of salicylate and related compounds in SIR mode. They were separated well by the GC. Full mass spectra of these derivatives are shown in Figure 2A–I. The RT and the specific ions chosen for measurement of each compound in SIR mode (mostly $M^+$-15) are listed in Table 1.

Standard curves were obtained by measuring the response ratio of each derivative to that of the internal standard, and were plotted *vs* their known concentrations. Standard curves were linear for all individual compounds in the concentration ranges investigated (0.1–10 nmol) with correlation coefficients $r > 0.999$. The absolute recoveries of all the compounds from plasma were in the range of 90–102%. Calculated results were based on triplicate experiments. The detection limit was between 50 fmol to 1 pmol per 1 μL injection.

Table 2 presents the precision data of the assay for the determination of catechol, 2,3-DHBA, and 2,5-DHBA. The precision of the method was assessed using 10 replicate samples of 5 separate experiments. The within-run and between-run coefficients of variation were between 4.6–9.1%.

TABLE 1
Retention Time (RT) adn Specific Ions of Mass Spectra of TMS Derivatives of Salicylate Hydroxylation Products and Related Aromatic Compounds

| Compound | Molecular Ion | Specific Ion* | RT (min) |
|---|---|---|---|
| Benzoic acid (ring-D5) | 199 | 184 | 7.46 |
| Catechol | 254 | 254† | 8.37 |
| Salicylic acid | 282 | 267 | 11.15 |
| Acetylsalicylic acid | 252 | 195‡ | 11.46 |
| 2,3-Dihydroxybenzoic acid | 370 | 355 | 15.03 |
| 2,6-Dihydroxybenzoic acid | 370 | 355 | 15.40 |
| 2,5-Dihydroxybenzoic acid | 370 | 355 | 15.48 |
| 2,4-Dihydroxybenzoic acid | 370 | 355 | 16.18 |
| Salicyluric acid | 339 | 324 | 20.49 |

* For most compounds, the specific ion for SIR is $M^+$-15, except as indicated.
† $M^+$.
‡ $M^+$-57.

## Discussion

Aspirin (ASP) is a commonly used analgesic, antipyretic, and anti-inflammatory agent in humans. ASP has also been found to interfere with platelet aggregation and is now approved by the U.S. Food and Drug Administration as a therapeutic substance for reducing the risk of transient ischemic attacks from stroke. After ingestion, a substantial amount of ASP is hydrolyzed to SA by esterases. SA is further metabolized to SUA, gentisic acid (GA, or 2,5-DHBA), gentisuric acid (GU), salicyl acyl glucuronide (SAG), and salicyl phenolic glucuronide (SPG) (15).

About 60% of SA remains unmodified and can undergo nonenzymatic addition reactions with hydroxyl radicals. Salicylate attacked by hydroxyl radicals produces characteristic hydroxylation products, namely, 2,3-DHBA, 2,5-DHBA, and catechol (16,17). Aromatic hydroxylation assays were first used in biological systems by Halliwell (10). These entail the addition of an aromatic compound to a reaction mixture producing hydroxyl radicals, and measurement of the formation of hydroxylated products. 2,5-DHBA can also be generated from SA enzymatically by cytochrome P-450 *in vivo,* but 2,3-DHBA is thought to be produced only by hydroxyl radical attack on salicylate. Catechol is formed by decarboxylation (18,19). Benzoate decarboxylation has been also used in assays for hydroxyl radicals *in vitro* (20).

Salicylate reacts with hydroxyl radicals with a rate constant of about $5 \times 10^9$ to $10^{10}$ $M^{-1}s^{-1}$ (21). Many techniques have already been used to evaluate the hydroxylated compounds in both *in vitro* and *in vivo* studies. The spectrophotometric and spectrofluorometric methods were adequate for *in vitro* studies, where high concentration of hydroxyl radicals are usually formed. More recently, HPLC techniques with UV detection or electrochemical detection became popular for *in vivo* studies (13,14). There is increasing evidence of 2,3-DHBA and/or 2,5-DHBA production (measured as concentration, or as a ratio to salicylate) after ASP or salicylate administration under conditions of oxidative stress, such as exposure to 100% oxygen (22), treatment with the redox-cycling drug doxorubicin (23), rheu-

TABLE 2
Within- and Between-Run Precision of the Assay for Catechol, 2,3-DHBA, and 2,5-DHBA Determination

| | Within-Run Precision (n = 10) | | | Between-Run Precision (n = 5) | | |
|---|---|---|---|---|---|---|
| | Catechol | 2,3-DHBA | 2,5-DHBA | Catechol | 2,3-DHBA | 2,5-DHBA |
| Mean | 90.2 | 93.5 | 94.2 | 91.5 | 94.9 | 101.8 |
| SD | 4.7 | 4.3 | 4.6 | 4.9 | 7.1 | 9.3 |
| CV (%) | 5.2 | 4.6 | 4.9 | 5.4 | 7.5 | 9.1 |

matoid arthritis (24), myocardial infarction (25), cerebral ischemia (26), diabetes (27), lung and kidney injuries (28,29), etc.

One of the drawbacks of using ASP as a probe is its inhibitory effect on cyclooxygenase, which can affect inflammatory responses. Because inflammation is associated with ROS production, inhibiting cyclooxygenase to measure hydroxyl radical production may give an uninterpretable response in such circumstances. Inhibition of platelet function by ASP could also underestimate hydroxyl radical production by the cyclooxygenase pathway (30). Salicylate has fewer pharmacological effects than ASP and direct administration of sodium salicylate could overcome some of these concerns.

Using our GC-MS method, the salicylate hydroxylation products and related aromatic compounds can be separated well and specifically quantified with high sensitivity. We have incubated normal human fibroblasts with 2 μM salicylate for 2 h, and measured the hydroxylation products. We were able to detect baseline values of catechol, 2,3-DHBA, and 2,5-DHBA (70–200 pmol/mg protein), and a significant increment after exposure to oxidative agents such as menadione or doxorubicin (data not shown). Preliminary experiment in this laboratory also showed increased salicylate hydroxylation products in ischemic and reperfused rabbit heart (data not shown). We believe the GC-MS assay presented here provides a convenient and sensitive method by which hydroxyl radicals may be detected and quantified both *in vivo* and *in vitro*. This procedure can also be applied to the pharmacological study of ASP and its metabolites.

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