Rapid High-Performance Liquid Chromatographic Assay for Salicylic Acid in Plasma Without Solvent Extraction

Charles Coudray[1,*], **Catherine Mangournet**[1], **Sophia Bouhadjeb**[1], **Henri Faure**[1], and **Alain Favier**[1,2]

[1]Laboratoire de Biochimie C, Centre hospitalo-universitaire de Grenoble, BP 17 X, 38034 Grenoble, France; [2]Groupe de Recherche et d'Etude sur les pathologies oxidatives (GREPO), Laboratoire de Biochimie pharmaceutique, UFR de Pharmacie, Domaine de la Merci, 38700 La Tronche, France

Abstract

The in vivo measurement of highly reactive free radicals, such as the hydroxyl radical (°OH), in humans is very difficult, if not impossible. Specific markers, based on the ability of °OH to attack aromatic molecules and produce hydroxylated compounds, are under investigation. In vivo radical metabolism of salicylic acid produces two main hydroxylated derivatives: 2,3- and 2,5-dihydroxybenzoic acid (DHBA). The measurement of 2,3-DHBA, following oral administration of salicylic acid or its acetylated form (aspirin), is proposed for the assessment of in vivo oxidative stress. The intensity of oxidative stress is a function of the ratio of dihydroxylated derivatives to salicylic acid rather than the absolute dihydroxylated derivatives levels. Consequently, a simple, accurate, and sensitive assay of the salicylic acid level in plasma is needed to investigate the in vivo free radical production. In this work, a rapid and sensitive method is presented that is useful for the quantitation of salicylic acid in biological fluids. The methodology uses high-performance liquid chromatography with spectrophotometric detection for the identification and quantitation of salicylic acid without organic extraction. A detection limit of less than 5 µmol is achieved with spectrophotometric detector responses that are linear over at least 6 orders of magnitude. Plasma concentrations of salicylic acid determined by the present technique are reported following the administration of 1000 mg aspirin in 20 healthy subjects.

Introduction

Reactive oxygen species have been implicated in a number of diseases (1–5) and in the physiological effects of aging (6,7). The in vivo measurement of highly reactive free radicals, such as the °OH radical, in humans is difficult. Recently, some direct methods have been proposed to identify the in vivo formation of the °OH radical. Nearly all are based on the ability of °OH to attack the benzene rings of aromatic molecules and to produce hydroxylated compounds that can be measured directly (8–15). A suitable aromatic compound for this use in humans is sali-

cylic acid (SA), or its acetylated form, aspirin.

Aspirin (O-acetyl salicylic acid, ASA) is an analgesic and anti-inflammatory agent commonly used by humans (15). After ingestion, a substantial amount of ASA is hydrolyzed to SA by esterases in the gastrointestinal tract and the liver (16). SA reaches its peak in plasma from about 0.5 to 2.0 h after oral intake. About 60% of SA remains unchanged and can undergo °OH attack to produce the following three products: 2,3-dihydroxybenzoate (DHBA), 2,5-DHBA, and, to a lesser extent, catechol (17,18). 2,3-DHBA appears to be a useful marker of in vivo °OH production when 2,3-DHBA to SA ratios are considered (19). Consequently, a simple, accurate, and sensitive assay of SA concentration in plasma is needed to investigate in vivo free radical production.

Several reports have appeared in the literature concerning the determination of SA concentrations. Methods to determine concentrations of SA in biological materials were developed in order to conduct pharmacokinetic studies of ASA and its metabolites (16,20). Some of the methods employed include: colorimetry, thin-layer chromatography, fluorimetry, gas chromatography, and high-performance liquid chromatography (HPLC) (21–29). The method developed in this work enables the reliable estimation of SA in plasma and, as a result, reinforces the investigation of the in vivo oxidative stress in humans.

Experimental

Chemicals

Aspirin (Aspégic®) was obtained from Synthelabo (Le Plessis Robinson; France), and sodium SA was obtained from Merck (Darmstadt, Germany). 2,3-DHBA, 2,5-DHBA, 2,4-DHBA, 2,6-DHBA, and 3,4-DHBA were purchased from Sigma (Paris, France). Acetonitrile, methanol, trisodium citrate, sodium acetate, ether, and ethyl acetate were obtained from Prolabo (Lyon, France). All other chemicals were analytical reagent grade and were used without further treatment. Deionized distilled water was used throughout the experiment.

---

* Author to whom correspondence should be addressed.

HPLC instrumentation

The chromatographic system was obtained from Kontron Instruments (Rotkreus, Switzerland) and consisted of two solvent-delivery pumps (Model T414); a sample injector (Kontron 234); an analytical stainless steel column packed with a 5-µm ultrasphere octadecyl silane reversed phase (150 × 4.6-mm i.d.) (Alltech; Paris, France); a guard column packed with 10-µm spheri-10 RP18 (30 × 4.6-mm i.d.) (Alltech); and an HPLC multiwavelength detection system (Model 430, Kontron). The system was controlled by Data System 450 (Kontron). The baseline was automatically adjusted to 0 at the beginning of each injection. The detector was programmed at 295 nm to determine SA. The detector sensitivity was set at 0.05 AUFS. Dihydroxybenzoic acids were assayed by an HPLC–electrochemical detection method as previously described (30).

Methods

The initial method of preparing the salicylic acid assay was previously described (31). Aliquots (400-µL) of standard solutions or plasma samples were mixed with 100 µL of 500µM 3,4-DHBA (internal standard) and acidified with 75 µL concentrated HCl (35%) in 10- × 70-mm glass tubes. The samples were vortex mixed for 30 s. Ether (3 mL) was added, and then the solution was extracted for 2 min. The tubes were centrifuged at 1600 × g for 15 min, and 2 mL of ether phase was dried under a nitrogen steam. The dry residue was reconstituted in 200 µL of mobile phase, and 50 µL was injected into the column. The mobile phase consisted of 30 mmol/L each of sodium citrate and acetate at pH 4.8. The flow rate was 1 mL/min, and detection was at 305 nm. Standard curves were drawn using peak-to-area ratios. Saline and plasma blanks were analyzed with each set of standards. The final concentrations used for calibration curves were as follows: 0, 62.5, 125, 250, 500, and 1000 µmol/L of SA.

Evaluation of the initial method

Linearity

The linearity of responses to different concentrations of SA (62.5–1000 µmol/L) and of 2,5-DHBA (2.5–40 µmol/L) was tested. The coefficients of variation (CV) and correlation coefficients (r^2) were then calculated.

Reproducibility

The reproducibility of the assay was determined by carrying out 20 replicate analyses of human plasma spiked with 500 µmol/L SA and 40 µmol/L 2,5-DHBA. Coefficients of variation were then determined.

Optimization procedures

Extraction procedure

An alternative procedure without organic extraction was examined. The volume of blood sample, the protein precipitation protocol, and the filtration and dilution procedures were studied.

Choice of eluent

The optimal composition, the pH of the eluent, and the flow rate were determined. The retention time, the separation of peaks, and the peak-to-noise ratio were recorded.

Choice of internal standard

Three potential internal standards, 2,4-DHBA, 2,6-DHBA, and 3,4-DHBA, were compared for optimum response and elution time.

Preparation of standard curves

Saline and plasma, which were found to have no detectable amount of SA by the present method, were used to make up solutions with concentrations that ranged from 62.5 to 1000 µmol/L SA. Stock solutions of 0.1 mol/L SA were found to be stable for at least 2 months when stored at 4°C and protected from light.

Analytical data

The detection limit was determined according to the method of Gatautis and Pearson (32). Samples with SA concentrations of 3 to 5 times the mean noise level (6.25 µmol/L) were measured 30 times. The detection limit was calculated using the formula $(2 \times SD \times C)/A$, where A is the mean relative area, SD is the corresponding standard deviation, and C is the concentration of the tested solution.

The linearity was established with correlation coefficients. Calibration solutions of SA (30–32,000 µmol/L) were determined. The coefficients of variation and of correlation were then calculated.
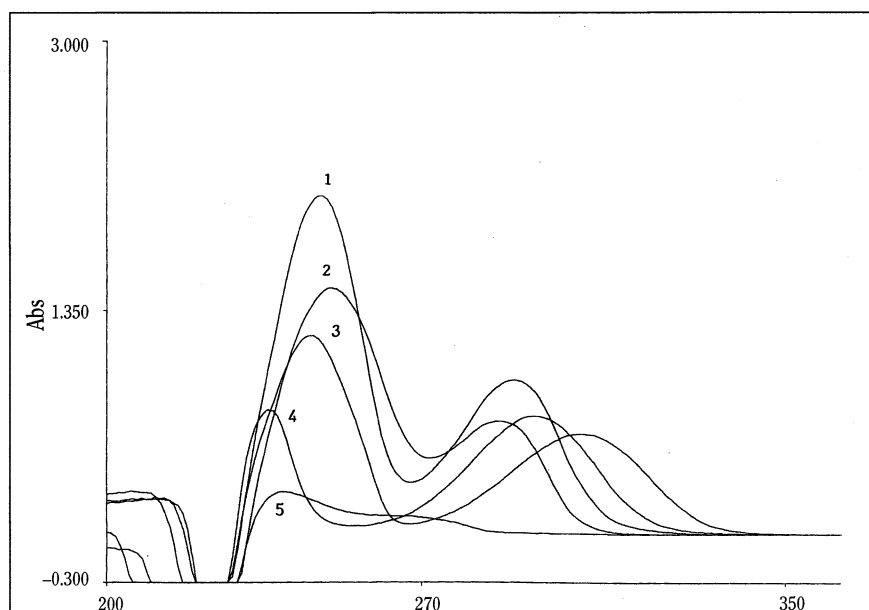
The precision was determined according to the ValTec pro-

Figure 1. Spectrophotometric scans of salicylic acid and its hydroxylated products. Solutions containing 200 µmol/L of all studied substances in buffer (citrate–acetate, 30mM:30mM, pH 5.45) were tested on a Uvikon instrument. Spectra are as follows: 1, 2,4-DHBA; 2, 3,4-DHBA: 3, 2,6-DHBA; 4, salicylic acid; and 5, aspirin.

tocol (33). Intra- and interassay reproducibility were conducted on pooled samples of human plasma with known concentrations of SA (600 and 1200 μmol/L) for interassay reproducibility and concentrations of 200, 400, 600, 800, and 1000 μmol/L for intra-assay reproducibility.

The accuracy was also evaluated in addition recovery studies. Known amounts of SA (333 and 1000 μmol/L) were added to saline and to pooled plasma. The preparations were then analyzed in 15 replicates. The peak areas thus obtained were converted to concentration values using appropriate standard curves, and the results obtained from plasma and saline were compared. The ratio of the concentration of SA in plasma to the corresponding concentration in saline was used as an index of recovery.

The specificity of the method was studied by assaying plasma samples from subjects before and after receiving ASA. Comparison of the retention times with those of standards established the peak identity.

Biological applications

To show the applicability of the proposed method, salicylic acid concentrations in the blood of 20 human subjects were measured after a single oral dose of 1000 mg of soluble ASA in 150 mL water. The doses were prepared just prior to administration. Blood samples (7 mL) were drawn from the antecubital vein into heparinized tubes with a vacutainer system before and 2 h after ASA administration.

Statistics

The PCSM statistic program (Dalta Soft; Meylan, France) was used in this study. Normal distribution of data was first verified by using the Kolmogorov–Smirnov test. Statistical analyses were conducted using a two-tailed, unpaired student t-test and linear regression. Differences between groups were considered to be significant when the value of p was less than 5%. Data are represented as mean plus standard deviation.

Results

Determination of optimal absorption and molecular extinction coefficients

Standard solutions of SA and its hydroxylated products at concentrations of 200 μmol/L were prepared in buffer, and spectra between 200 and 350 nm were recorded against buffer. As shown in Figure 1, SA absorption is maximal at 295 nm, whereas that of hydroxylated products is between 287 and 305 nm. For optimal sensitivity, the chromatograms for SA determination were recorded at 295 nm. Molecular extinction coefficients were 3350 (295 nm), 4635 (290 nm), 3015 (305 nm), 3380 (287 nm), and 521 (270 nm) M-1Cm-1 for SA, 2,4-DHBA, 2,6-DHBA, 3,4-DHBA, and aspirin, respectively.

Evaluation of the initial method

When the initial method was applied, the chromatograms shown in Figure 2 were obtained. The complete separation of a standard mixture containing SA, 2,5-DHBA, and the internal

standard (3,4-DHBA) or a plasma sample spiked with SA, 2,5-DHBA, and the internal standard was achieved. The flow rate for the HPLC mobile phase was 1 mL/min, and the chromatograms were recorded at 305 nm. The three peaks were well separated within 20 min with the following retention times: 3,4-DHBA, 3.70; 2,5-DHBA, 6.05; and SA, 15.50 min. In order to evaluate the analytical performance of the technique, its linearity and reproducibility were studied.

Linearity

In order to determine linearity, calibration standard solutions of 5 degrees of amplitude (SA, 62.5–1000 μmol/L; 2,5-DHBA, 2.5–40 μmol/L) were determined several times. The
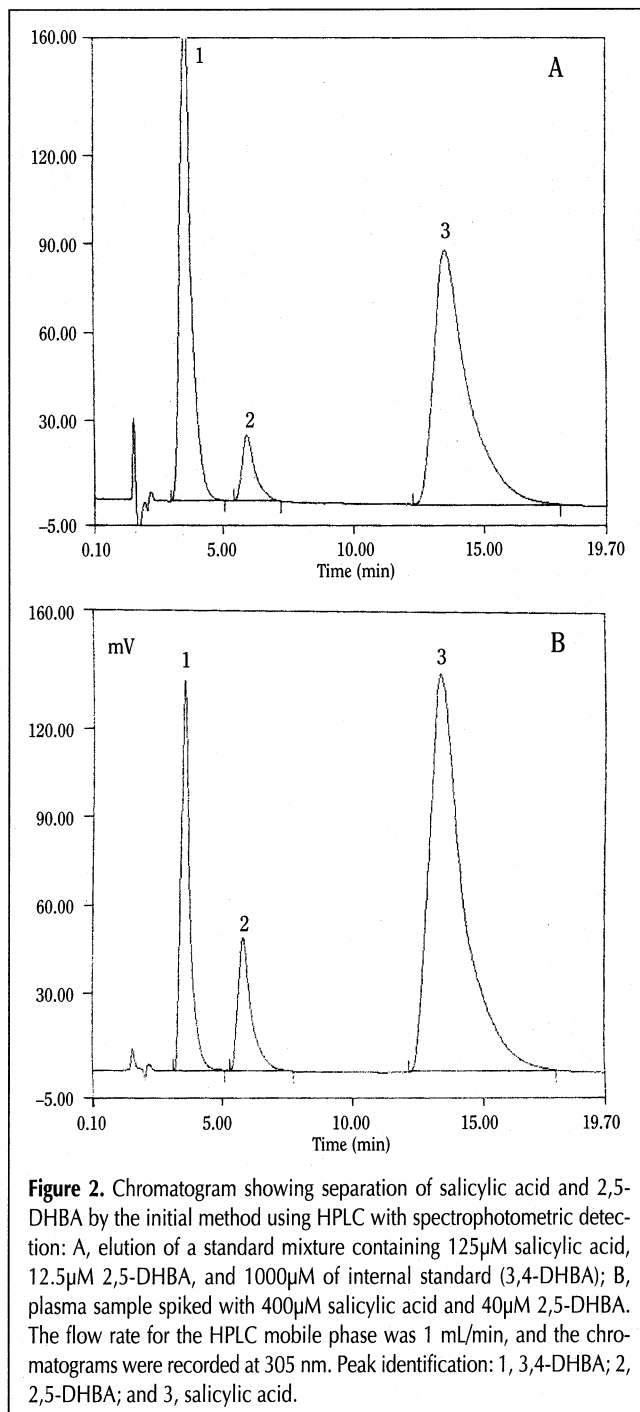

Figure 2. Chromatogram showing separation of salicylic acid and 2,5-DHBA by the initial method using HPLC with spectrophotometric detection: A, elution of a standard mixture containing 125μM salicylic acid, 12.5μM 2,5-DHBA, and 1000μM of internal standard (3,4-DHBA); B, plasma sample spiked with 400μM salicylic acid and 40μM 2,5-DHBA. The flow rate for the HPLC mobile phase was 1 mL/min, and the chromatograms were recorded at 305 nm. Peak identification: 1, 3,4-DHBA; 2, 2,5-DHBA; and 3, salicylic acid.

linear regression and correlation coefficients were then calculated. Surprisingly, the linearity of 2,5-DHBA was always excellent ($y = 2.2791x + 0.6289$; $r^2 = 0.998$; CV = 1.2%), whereas the salicylic acid assay showed poor linearity ($y = 4.0585x + 90.8362$; $r^2 = 0.876$; CV = 21.3%) (where y is concentration in micromoles and x is peak area in millivolts times minutes). However, when standard solutions were injected directly, without any sample preparation, linear responses were obtained for the two compounds (SA and 2,5-DHBA) with correlation coefficients of 0.999 and 0.999 respectively and coefficients of variation of 0.9% and 0.8%, respectively, as shown in Figure 3.

Reproducibility

The reproducibility of the assay was checked by several determinations of human plasma spiked with 500 µmol/L SA and 50 µmol/L 2,5-DHBA on the same day. The following coefficients of variation were obtained: SA, 18.3% and 2,5-DHBA, 2.9%. Excellent reproducibility was noted for the 2,5-DHBA assay, but the SA determination presented poor reproducibility.

Many tests were undertaken to ameliorate the unsatisfactory analytical performance of the salicylic acid assay. The results of extraction using ethyl acetate were compared with those using ether, and the influence of the solvent was studied. The dry residue was taken up after evaporation by various solutions (mobile phase with different pHs, water, methanol,

NaOH between 0.001 and 0.50M, and HCl 0.1M–water). The extraction and the vortex mixing times necessary for extraction and solubilization of the SA were also noted. All our attempts to improve the analytical performance of the SA assay in terms of linearity, reproducibility, and recovery were unsuccessful. Thus, the method was abandoned in order to develop an alternative one without the extraction step.

Optimization steps

Sample treatment

Different procedures of deproteinization were studied (acetonitrile or absolute ethanol). Deproteinization of plasma was undertaken by mixing two volumes of the tested solvent with one volume of sample and one volume of internal standard (dissolved in buffer). After 10 min of centrifugation, acetonitrile seemed to achieve efficient deproteinization, and the supernatant obtained was clear and appeared to be protein free. The supernatants were filtered, and 50 µL of filtrate was injected directly into the HPLC column. However, the chromatograms were difficult to interpret when acetonitrile was used. Two important unidentified peaks eluted close to the SA and internal standard peaks. Ethanol was thus selected for use in the deproteinization procedure, even though the chromatogram presented a small unidentified peak near the SA peak. The response of the detector was important, so in order to minimize
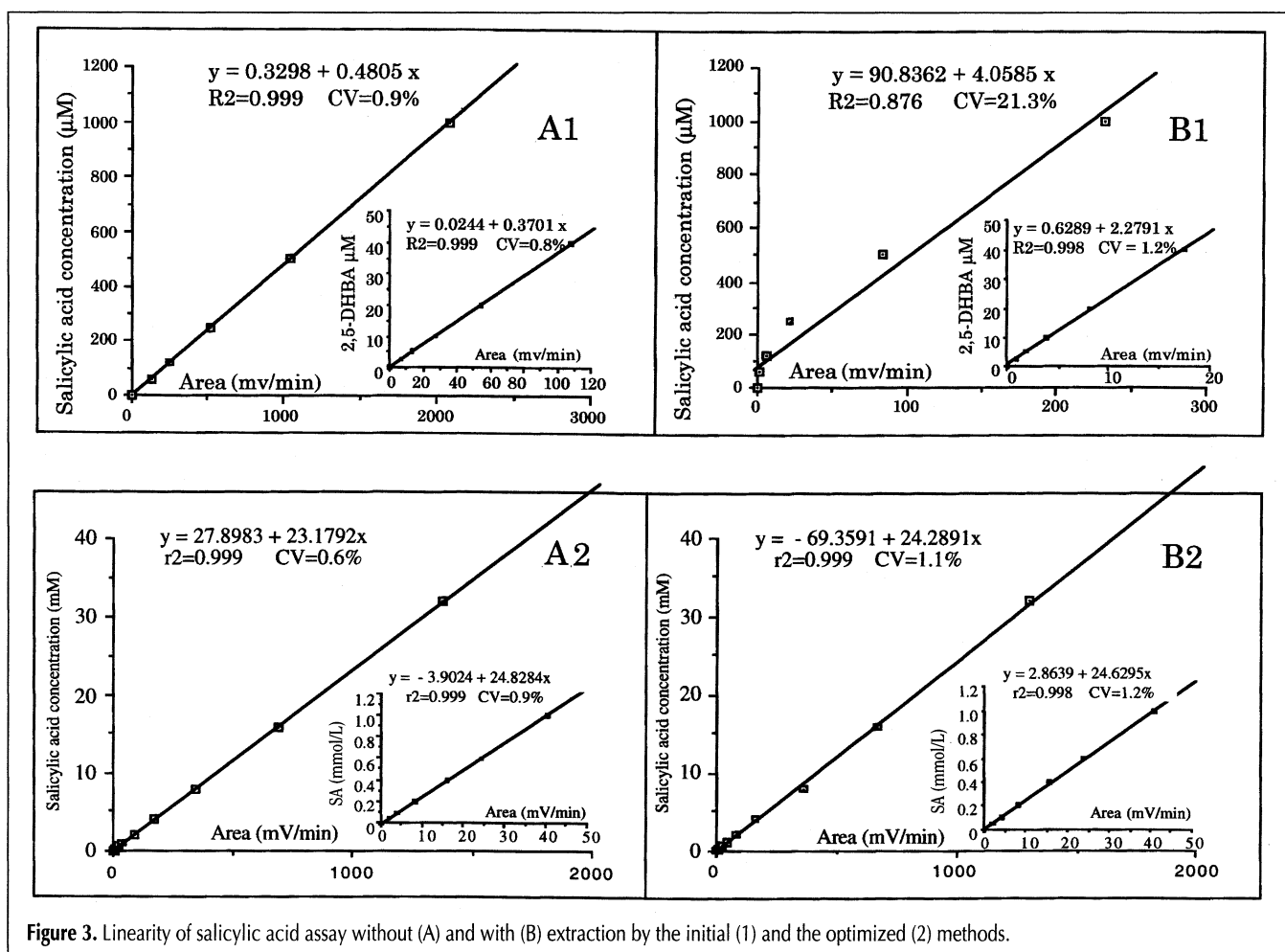

Figure 3. Linearity of salicylic acid assay without (A) and with (B) extraction by the initial (1) and the optimized (2) methods.

the possibility of column overload, different dilutions of the supernatant were used before or after filtration. The results showed that a dilution of 1:20 was appropriate, and the results were identical whether this dilution was performed before or after filtration.

Choice of eluent

An isocratic delivery system consisting of a single eluent containing sodium acetate–trisodium citrate (30mM:30mM) buffer (pH 5.40–5.45) was used. The different standards (SA, and 3,4-DHBA) were injected separately in order to examine the peak shapes and retention times. A serum sample was then injected and examined. The retention time of SA was more than 40 min, and different percentages of methanol were tested to shorten it. A mobile phase consisting of 85% buffer and 15% methanol was selected to achieve maximum separation and sensitivity (Table I). Flow rates between 0.5 and 1.5 mL/min were studied. A flow rate of 1.0 mL/min gave an optimal signal-to-noise ratio with a reasonable separation time (Table II). Moreover, isocratic elution allowed a minimum eluent consumption, reduced the time period between runs, and extended column life.

Choice of internal standard

Different substances (3,4 DHBA, 2,4-DHBA, and 2,6-DHBA) previously used by other investigators were studied. In our assay conditions, the internal standards 3,4-DHBA and 2,4-DHBA were eluted rapidly and were indistinguishable from the unidentified peak in the plasma samples. However, the internal standard 2,6-DHBA was eluted sufficiently after the SA peak and was used in the following protocols.

Definitive protocol

Aliquots (100 µL) of standard solutions or plasma samples were mixed with 100 µL of 2.5 µmol/L 2,6-DHBA and deproteinated by 200 µL ethanol in 1.5-mL polypropylene conical Eppendorf micro test tubes. The samples were vortex mixed for 2 min. The tubes were then centrifuged at $1600 \times g$ for 15 min, and 50 µL of the supernatant was diluted with 950 µL of mobile phase. The diluted solution was then filtered on 0.45-µm filters (Alltech), and 50 µL of solution was injected into the column. The mobile phase consisted of 30 mmol/L sodium citrate and acetate at pH 5.45 and methanol (85:15). The flow rate was 1 mL/min, and detection was at 295 nm. Standard curves were constructed from measurements of peak-area ratios. Saline and plasma blanks were analyzed with each set of standards. The concentrations were as follows: 0, 62.5, 125, 250, 500, and 1000 µmol/L of SA.

Analytical performance

Detection limit

The detection limit was found to be 3.89 µmol/L in the biological sample when calculated according to the formula of Gatautis and Pearson (32). It corresponds to an injected quantity of 12.43 pmol SA. These limits demonstrate the excellent sensitivity of the proposed method for SA. These ranges allow its determination after oral ingestion of ASA in moderate doses in all patients who risk intensive oxidative stress.

Linearity

Calibration curves of the SA standard solutions prepared in water or in plasma were plotted over the range 30–32,000 µmol/L. The response of SA remained linear up to the highest concentration tested (32,000 µmol/L). The regression line of SA was also linear when prepared in water ($y = 27.8983 + 23.1792x$; $r^2 = 0.999$) or in plasma ($y = -69.359 + 24.289x$; $r^2 = 0.999$) (where y is concentration in micromoles and x is peak area in millivolts times minutes). The coefficients of variation between assays were 0.6% and 1.1%, respectively, as judged by changes in the slope of different calibration curves. Figure 3 shows the calibration curves for SA and the corresponding correlation coefficients and CVs.

Table I. Effect of Mobile Phase Composition on Retention Time (in minutes) and Separation of Salicylic Acid (SA) and its Potential Internal Standards (IS)*

	100% buffer	5% methanol	10% methanol	15% methanol	20% methanol
Salicylic acid	16.53 ± 0.09	12.24 ± 0.06	9.41 ± 0.05	6.02 ± 0.03	4.39 ± 0.02
Internal standard	34.3 ± 0.17	23.42 ± 0.11	16.52 ± 0.09	9.81 ± 0.07	6.61 ± 0.05
SA/IS	0.481	0.521	0.570	0.614	0.664

* Buffer consisted of sodium citrate–sodium acetate (30mM:30mM) at pH 5.45. The flow rate was 1 mL/min. The internal standard was 2.6-DHBA.

Table II. Effect of Flow Rate (in milliliters per minute) on Retention Time (in minutes)*

		1.50	1.25	1.00	0.75	0.50
Unidentified 1	in water	–	–	–	–	–
	in plasma	1.37	1.65	2.00	2.68	3.87
3,4-DHBA	in water	1.59	1.91	2.29	3.08	4.39
	in plasma	1.58	1.90	2.30	3.10	4.47
2,4-DHBA	in water	1.68	2.10	2.51	3.41	4.93
	in plasma	1.70	2.07	2.53	3.40	4.95
Unidentified 2	in water	–	–	–	–	–
	in plasma	4.14	4.99	6.11	8.24	12.0
Salicylic acid	in water	4.78	5.77	6.98	9.49	13.8
	in plasma	4.79	5.76	7.00	9.50	13.8
2,6-DHBA	in water	7.73	9.29	11.3	15.3	22.3
	in plasma	7.74	9.31	11.4	15.4	22.4

* Mobile phase consisted of buffer–methanol (85:15). The salicylic acid concentration was 600 µmol/L in water or in human plasma. The internal standards were 1mM 3,4-DHBA, 1mM 2,4-DHBA, and 1mM 2,6-DHBA.

Precision

The variability of repeated injections of the same plasma sample was less than 1.0% (15 determinations). The overall analytical variability among 15 identical samples measured the same day was less than 2% (within-run precision). The variation between different days was less than 4% (between-run precision). The complete results are given in Table II. The between-run and recovery assays were conducted during a 3-week period. During this period, the stability of SA was good, as shown by the correlation coefficients.

Accuracy

The response of SA in 100-μL plasma spiked with varying amounts of SA standard (333 and 1000 μmol/L) was studied. Absolute recoveries of SA added at two different concentrations to plasma were satisfying, with a mean recovery of 96.2%. Almost 4% of SA was lost, perhaps stopped on filtration membranes or eliminated with plasma protein during deproteinization.

Salicylic acid in the plasma of healthy volunteers

Figure 4 shows a typical chromatogram of a blank plasma sample and a chromatogram of a plasma sample from a subject receiving ASA. The peaks of SA and the internal standard were well separated, and the retention times were as follows: SA, 7.2 min and 2,6-DHBA, 11.4 min. No interfering peaks were seen in blank plasma samples. Analysis of samples drawn before ASA administration shows that volunteers' plasma was free of SA. High concentrations of SA, reaching more than 800 μmol/L in some subjects, were observed in plasma samples that were obtained from the volunteers 2 h after aspirin administration.

Twenty laboratory staff (10 women and 10 men), aged 20 to 40 years (mean age, 28.2±5.22 years), participated in this study. Two hours after the administration of 1000 mg ASA, the mean SA concentration was 487 ± 116 (M ± SD). The level of 2,3-DHBA in plasma was 63.2 ± 23.8 (M ± SD). Plasma ASA levels were not determined in this study. As a result of individual variations in the pharmacokinetics of aspirin, the plasma level of salicylic acid could vary largely from one indi-

| Table III. Repeatibility and Reproducibility of the Method			
	Mean	Standard deviation	Coefficient of variation (%)
Repeatability (Within-run precision)			
Carried out in water (n = 15)			
Repeatability of the HPLC	603.1	4.11	0.7
Repeatability of the assay	598.3	8.31	1.4
Carried out in human plasma (n = 15)			
Repeatability of the HPLC	581.9	5.71	1.0
Repeatability of the assay	575.1	10.3	1.7
Reproducibility (Between-run precision)			
Carried out in water (n = 6)			
200 μmol SA/L	195.2	7.64	3.9
1000 μmol SA/L	992.8	30.78	3.1
Carried out in human plasma (n = 6)			
200 μmol SA/L	192.4	8.37	3.8
1000 μmol SA/L	955.7	33.36	3.5
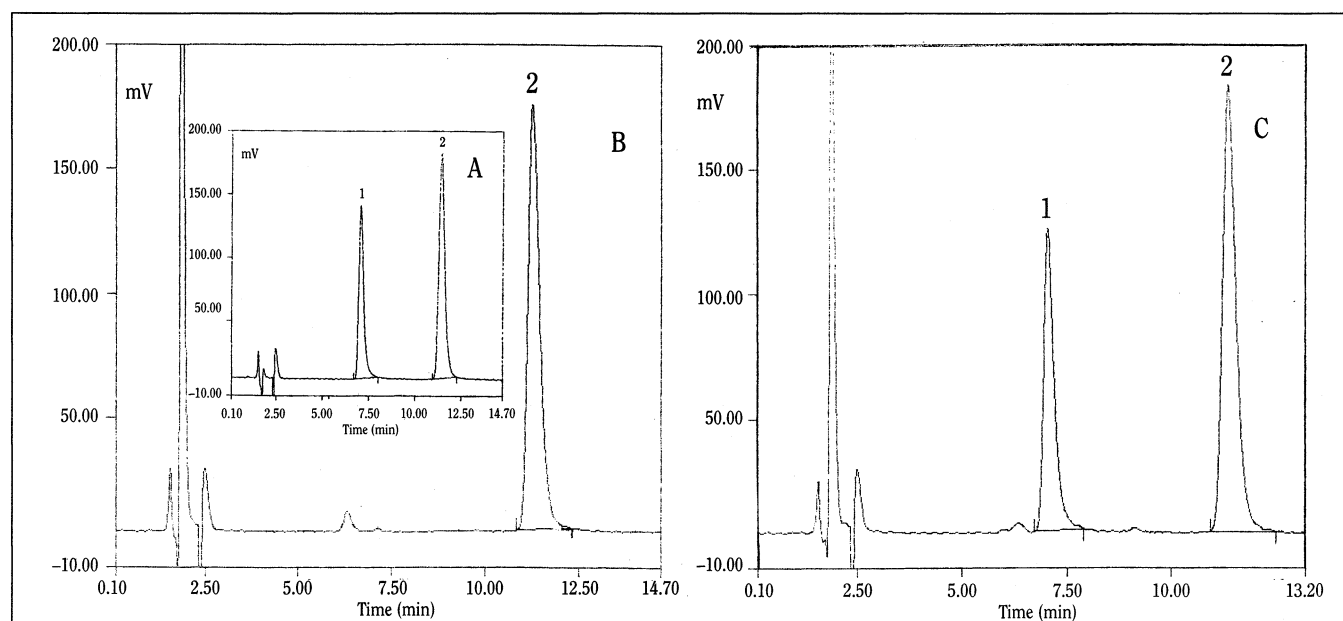
Figure 4. Chromatogram showing separation of salicylic acid with the modified method using HPLC with spectrophotometric detection: A, elution of a standard mixture containing 1mM salicylic acid and 2.5 mmol of internal standard (2,6-DHBA); B, plasma sample from a normal subject before 1000 mg ASA administration; C, plasma sample from the same subject 2 h after ASA administration. The flow rate for the HPLC mobile phase was 1 mL/min, and the chromatograms were recorded at 295 nm. Peak identification: 1, salicylic acid and 2, 2,6-DHBA (internal standard).

vidual to another (344–868 µmol/L). Consequently, the concentration of DHBA in plasma should always be corrected to that of the concentration of SA in plasma.

Discussion

Direct evidence for the formation of free radicals in oxidative processes exists, but the causal relationship between free radicals and damage is still lacking. The major reason is that free radicals are present in minute amounts in the tissues. They are highly reactive, and they have short lives (34,35). Specific markers, based on the ability of °OH to attack aromatic molecules and to produce hydroxylated compounds, are under investigation (36). The measurement of 2,3-DHBA following oral administration of SA or ASA has been proposed for assessment of in vivo oxidative stress (37). The intensity of oxidative stress is a function of the ratios of dihydroxylated derivatives to salicylic acid rather than the absolute dihydroxylated derivative levels (38–41). Therefore, a simple, sensitive, and accurate method is required for SA determination.

Many techniques have already been used to evaluate SA and its metabolites in biological fluids. Spectrophotometric assays were shown to be appropriate for in vitro studies where large amounts of SA are usually used or for aspirin intoxication. These techniques still lack accuracy and sensitivity. Assays of SA by gas chromatography have been reported (21,22). However, time-consuming chemical derivatization, such as silylation, was necessary. Many HPLC assays have been developed to separate and quantitate SA specifically and sensitively in pharmaceutical formulations (42) and biological fluids (23–29). However, they usually require solvent extraction, including the use of benzene, which is toxic.

We describe a rapid determination of SA in human plasma without organic extraction. The detection limit, calculated according to Gatautis and Pearson (32) was less than 5 µmol SA/L. If other calculation approaches are considered, such as the height of the baseline times 3, this limit could be lowered to less than 2 µmol SA/L. Of course, this detection limit is function of solvent purity, column quality, and apparatus equilibration. Thus, this method has adequate sensitivity. Under our experimental conditions, the calibration curve was perfectly linear up to at least 32,000 µmol SA/L (5376 mg/L). Coefficients of correlation were close to 1, and the coefficients of variation were less than 3%. The proposed protocol gives excellent within- and between-run precision with coefficients of variation of less than 4%. Finally, the percentage of recovery of the different concentrations of SA from spiked human plasma is more than 95% for 333 and 1000 µmol SA/L. This percentage is satisfactory.

For accurate determination of SA, at least two precautions are necessary. First, the blood samples must be collected, preferably into anti-hydrolysis compound-containing tubes (potassium fluoride) to prevent ASA from hydrolysis in human blood after sampling (27). This is especially true when samples are drawn between 0 and 90 min after ASA ingestion when the ASA level is high. Secondly, for techniques using organic ex-

traction steps, the extraction solvent must be evaporated in an ice water bath to prevent underestimation of SA concentration due to loss through sublimation (39).

In the present study, after an oral dose of 1000 mg of soluble ASA, the maximum concentration of SA in plasma is in the expected ranges. Two hours after ASA administration, the plasma SA level was 487 ± 116; 245–729 µmol/L (M ± SD; M ± 2.086 SD). Lo and Bye (23) reported levels of approximately 200 µmol/L 2 hours after an oral dose of 600 mg soluble ASA. Cham and co-workers (24) reported an SA concentration of 600 µmol/L 2 h after an oral dose of 21 mg/kg (1365 mg). Finally, Cham and co-workers (16) found that SA concentration reaches its maximum between 30 and 60 min (380 µmol/L) when 900 mg efflorescent ASA was ingested, and its concentration at 2 h was approximately 200 µmol/L. Because the level of 2,3-DHBA in plasma depends on that of SA, which could vary between individuals who ingest the same dose of aspirin, the concentration of 2,3-DHBA should be expressed as the 2,3-DHBA–SA ratio (in millimoles per mole). The presence of 2,3-DHBA in the plasma of healthy subjects after taking aspirin could be a result of the baseline rate of intracellular °OH formation from ionizing radiation (43) and in vivo Fenton reactions and by blood leukocytes (41).

Conclusion

A newly developed methodology is now available in which SA can be easily and specifically identified and quantitated with high sensitivity using HPLC coupled with spectrophotometric detection. The mobile phase was carefully selected to achieve a maximum separation in a minimum amount of time. The isocratic elution allows a minimum of downtime. The improved SA assay as described in the present paper provides a simple, rapid, and convenient method by which SA may be detected and quantitated in vivo. Moreover, the small plasma requirement (0.1 mL) permits determinations in plasma from infants and young children.

References

1. J. Di Guiseppi and I. Fridovich. The toxicology of molecular oxygen. *CRC Crit. Rev. Toxicol.* **12:** 315–42 (1984).
2. B. Halliwell. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis (Review). *Br. J. Exp. Path.* **70:** 737–57 (1989).
3. B. Halliwell and C.E. Cross. Reactive oxygen species, antioxidants, and acquired immunodeficiency syndrome. *Arch. Intern. Med.* **151:** 29–31 (1991).
4. B. Halliwell and J.M.C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219:** 114 (1984).
5. B. Halliwell, J.M.C. Gutteridge, and D.R. Blake. Metal ions and oxygen radical reactions in human inflammatory joint disease. *Philos. Trans. R. Soc. Lond. (Biol)* **311:** 659–71 (1985).
6. B.N. Ames. Endogenous oxidative DNA damage, aging and cancer. *Free Rad. Biol. Med.* **7:** 121–28 (1989).
7. D Harman. Free radicals in aging. *Mol. Cell. Biochem.* **84:** 155–61 (1988).

8. H. Kaur and B. Halliwell. Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals; measurement of hydroxyl radicals formation from ozone and in blood from premature babies using improved high performance liquid chromatography methodology. *Anal. Biochem.* **220:** 11–15 (1994).

9. A. Ghiselli, O. Lauranti, G. De Mattia, G. Maiani, and A. Ferro-Luzzi. SA hydroxylation as an early marker of in vivo oxidative stress in diabetic patients. *Free Rad. Biol. Med.* **13:** 621–26 (1992).

10. R.A. Floyd, R. Henderson, J.J. Watson, and P.K. Wong. Use of SA with high pressure liquid chromatography and electrochemical detection (LEC) as a sensitive measure of hydroxyl free radicals in adriamycin-treated rats. *Free Rad. Biol. Med.* **2:** 13–18 (1986).

11. M. Grootveld and B. Halliwell. Aromatic hydroxylation as a potential measure of hydroxyl radical formation in vivo. Identification of hydroxylated derivatives of SA in human body fluids. *Biochem. J.* **237:** 499–504 (1986).

12. M. Tubaro, G. Cavallo, M.A. Pensa, E. Natale, R. Ricci, F. Milazzotto, and E. Tubaro. Demonstration of the formation of hydroxyl radicals in acute myocardial infarction in man using SA as probe. *Cardiology* **80:** 246–51 (1992).

13. S.R. Powell and D. Hall. Use of SA as a probe for °OH formation in isolated ischemic rat hearts. *Free Rad. Biol. Med.* **9:** 133–41 (1990).

14. R. Udassin, I. Ariel, Y Haskel, N. Kitrossky, and M. Chevion. SA as an in vivo free radical trap: studies on ischemic insult to the rat intestine. *Free Rad. Biol. Med.* **10:** 1–6 (1991).

15. D.M. Woodbury and E. Fingl. Analgestic antipyretics, anti-inflammatory agents and drugs employed in the therapy of gout. In *The Pharmacological Basis of Therapeutics,* 5th ed. L.S. Goodman and A. Gilman, Eds. MacMillan, New York, NY, 1975, pp 325–58.

16. B.E. Cham, L. Ross-Lee, F. Bochner, and D.M. Imhoff. Measurement and pharmacokinetics of acetylsalicylic acid by a novel high performance liquid chromatographic assay. *Therap. Drug Monit.* **2:** 365–72 (1980).

17. B. Halliwell and M. Grootveld. The measurement of free radical reactions in humans: some thoughts for future experimentation. *FEBS Lett.* **213:** 9–14 (1987).

18. M. Grootveld and B. Halliwell. 2,3-Dihydroxybenzoic acid is a product of aspirin metabolism. *Biochem. Pharmacol.* **37:** 271–80 (1988).

19. B. Halliwell, H. Kaur, and M. Ingelman-Sunberg. Hydroxylation of SA as an early assay for hydroxyl radicals: a cautionary note. *Free Rad. Biol. Med.* **10:** 439–41 (1991).

20. J.R. Leonards. Presence of acetylsalicylic acid in plasma following oral ingestion of aspirin. *Proc. Soc. Exp. Biol. Med.* **110:** 304–308 (1962).

21. D. Schachter and J.G. Manis. SA and salicyl conjugates: fluorimetric estimation, biosynthesis and renal excretion in man. *J. Clin. Invest.* **38:** 800–807 (1958).

22. M.J. Rance, B.J. Jordan, and J.D. Nichols. A simultaneous determination of acetylsalicylic acid, salicylic acid and salicylamide in plasma by gas liquid chromatography. *J. Pharm. Pharmacol.* **27:** 425–29 (1975).

23. L.Y. Lo and A. Bye. Specific and sensitive method for the determination of aspirin and salicylic acid in plasma using reversed-phase high performance liquid chromatography. *J. Chromatogr.* **181:** 473–77 (1980).

24. B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff, and M. Rowland. Simultaneous liquid-chromatographic quantification of salicylic acid, salicyluric acid and gentisic acid in plasma. *Clin. Chem.* **25:** 1420–25 (1979).

25. U. Reidl. Determination of acetylsalicylic acid and its metabolites in biological fluids by high performance liquid chromatography.

J. Chromatogr. **272:** 325–31 (1983).

26. F.A. Ogunbona. Simultaneous liquid-chromatographic determination of aspirin and its metabolites in human urine. *J. Chromatogr.* **377:** 471–74 (1986).

27. R.J. O'Kruk, M.A. Adams, and R.B. Philp. Rapid and sensitive determination of acetylsalicylic acid and its metabolites using reversed-phase high performance liquid chromatography. *J. Chromatogr.* **310:** 343–52 (1984).

28. R.H. Rumble and M.S. Roberts. Determination of aspirin and its metabolites in plasma by high performance liquid chromatography without solvent extraction. *J. Chromatogr.* **225:** 252–60 (1981).

29. S.G. Owen, M.S. Roberts, and W.T. Friesen. Rapid high performance liquid chromatography assay for the simultaneous analysis of non-steroidal anti-inflammatory drugs in plasma. *J. Chromatogr.* **416:** 293–302 (1987).

30. C. Coudray, M. Talla, S. Martin, M. Fatôme, and A. Favier. High performance liquid chromatography electrochemical determination of SA hydroxylation products as an in vivo marker of oxidative stress. *Anal. Biochem.* **227:** 101–11 (1995).

31. R., Richmond, B. Halliwell, J. Chauhan, and A. Darbre. Superoxide-dependent formation of hydroxyl radicals: detection of hydroxyl radicals by the hydroxylation of aromatic compounds. *Anal. Biochem.* **118:** 328–35 (1981).

32. V.J. Gatautis and K.H. Pearson. Separation of plasma carotenoids and quantification of β-carotene using HPLC. *Clin. Chim. Acta* **166:** 195–206 (1987).

33. A. Vassault, D. Grafmeyer, C. Naudin, G. Dumont, M. Bailly, et al. Commission validation de techniques; protocole de validation de techniques (document B, stade 3). *Ann. Biol. Clin.* **44:** 686–92 (1986).

34. B. Halliwell and J.M.C. Gutteridge. Oxygen free radicals and iron in relation to biology and medicine; some problems and concepts. *Arch. Biochem. Biophys.* **246:** 501–14 (1986).

35. J.M.C. Gutteridge. Biological origin of free radicals, and mechanisms of antioxidant protection. *Chemico. Biol. Interactions* **91:** 133–147 (1994).

36. R.A. Floyd, J.J. Watson, and P.K. Wong. Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and SA hydroxylation products. *J. Biochem. Biophys. Meth.* **10:** 221–235 (1984).

37. A. Soren. Kinetics of SA in blood and joint fluid. *J. Clin. Pathol.* February/March: 173–78 (1973).

38. T. Obata, H. Hosokawa, and Y. Yamanaka. Effect of ferrous iron on the generation of hydroxyl free radicals by liver microdialysis perfusion of SA. *Comp. Biochem. Physiol.* **106C:** 629–34 (1993).

39. M.J. O'Connell and N.R. Webster. Hyperoxia and SA metabolism in rats. *J. Pharm. Phamacol.* **42:** 205–206 (1990).

40. T. Onodera and M. Ashraf. Detection of hydroxyl radicals in the post-ischemic reperfused heart using SA as a trapping agent. *J. Mol. Cell. Cardiol.* **23:** 365–70 (1991).

41. W.B. Davis, B.S. Mohammed, D.C. Mays, Z.W. She, J.R. Mohammed, et al. Hydroxylation of SA by activated neutrophils. *Biochem. Pharmacol.* **38:** 4013–19 (1989).

42. S.L. Ali. Application of gas–liquid chromatography and high-performance liquid chromatography to the analysis of trace amounts of salicylic acid, acetylsalicylic anhydride and acetylsalicylic acid in aspirin samples and aspirin formulations. *J. Chromatogr.* **126:** 651–63 (1976).

43. M. Grootveld and B. Halliwell. Aromatic hydroxylation as a potential measure of hydroxyl radical formation in vivo. Identification of hydroxylated derivatives of SA in human body fluids. *Biochem. J.* **237:** 499–504 (1986).