Anal. Chem. **2000,** *72,* 634−638

Determination of a Sulfur-Containing Drug in Human Plasma by an Improved Method for Sulfur Chemiluminescence Detection in Combination with Capillary Gas Chromatography

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An improved method for sulfur chemiluminescence detection in combination with capillary gas chromatography was established. The method was applied to the analysis of a sulfur-containing antiasthma drug, S-1452, and its nine metabolites in human plasma. The high selectivity and sensitivity of the method allowed accurate quantitation of trace levels of these compounds in human plasma with no interferences from the biological components present. To enable stable measurements and maintain reproducibility over a long period, the performance characteristics of a commercially available instrument were investigated. The gas seal in the injection port was found to easily corrode through interaction of the sulfur analyte with the metal gas seal. To prevent this, a disk made from an alloy of platinum and gold (60:40) was mounted on the gas seal. The reproducibility of the measurement was improved remarkably by using the disk. The use of compressed air of high purity significantly lowered the signal-to-noise ratio. The optical filter was kept clean by using a nickel catalyst to trap ozone in place of copper manganese oxide ($CuMn_2O_4$). These improvements raised the sensitivity and selectivity with the lower quantitation limits of 0.5−1.0 ng/mL in human plasma.

The ozone-based sulfur chemiluminescence detector (SCD) has been widely used as a highly sensitive and selective means of detecting sulfur-containing compounds in combination with capillary gas chromatography (CGC), high-performance liquid chromatography, or supercritical fluid chromatography,[1−8] to enable analyses of trace amounts of the element sulfur in quality evaluation of various compounds in petrochemicals,[9−11] foods,[12−14] and agricultural chemicals[15−17] and in environmental assessment.[18−20] Many of the CGC−SCD methods have been applied to analyze low molecular weight compounds including gaseous samples such as thiols, sulfides, and thiophenes. However, there have been no reports on the application of CGC−SCD to the determination of sulfur-containing drugs in biological samples. Three problems lie in the way of application of a commercially available CGC−SCD system under the generally used conditions to precise determination in complex matrixes such as human plasma: first, corrosion of the metal seal at the injection port; second, adherence of contaminant on the UV filter in the chemiluminescence reaction cell; and third, purity of the gases (air and hydrogen). We have solved the above problems by modification of the system to establish an assay method for an antiasthma drug S-1452 and its nine metabolites in human plasma.

EXPERIMENTAL SECTION

Instrumentation. A Hewlett-Packard (HP) model 5890 series II gas chromatograph equipped with a flame ionization detector (FID) was used. The FID was adapted to the combustion of the effluent and operated under hydrogen-rich conditions without connecting the detection system. A Sievers Research (Boulder, CO) model 350N sulfur chemiluminescence detector was connected to the FID on which the interface assembly was mounted through the transfer line. A ceramic probe was fixed in the FID interface assembly to keep the distance to the flame jet at 6.0 mm. An oil filtration system, model UFO 003 (Sinku Kiko, Yokohama, Japan), was connected to an Edwards model E2-M5 (West Sussex, U.K.) vacuum pump through the oil drain port. Fomblin oil of a specific gravity more than 1.85 and of low viscosity was purchased

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10.1021/ac990927l CCC: $19.00
Published on Web 01/05/2000

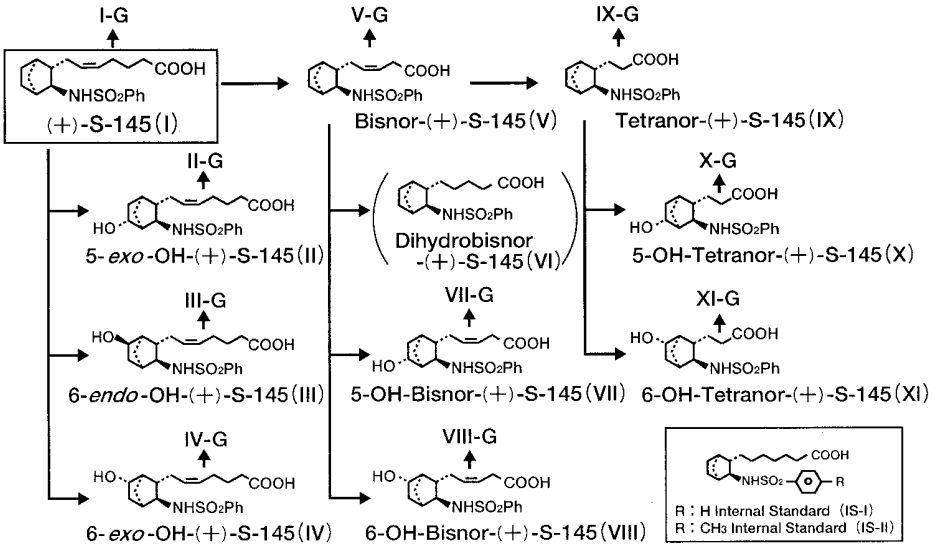

Figure 1. Structures of (+)-S-145, its metabolites, and internal standards: G next to the compound numbers means the glucuronide.

from Ausimont (Via San Pietro, Italy) and placed in the pump. The flow rate of the oil was adjusted to 0.6 mL/min. Copper manganese oxide ($CuMn_2O_4$, hopcalite)[21] filling the ozone trap was replaced with a pellet-shaped nickel catalyst (3 mm in both diameter and length) generally used in chemical industries.[22] The catalyst type N112 was purchased from GL Sciences (Tokyo, Japan). Each gas, hydrogen, helium, and air, was passed through a Gas Clean Filter system (Chrompack, Holland). The flow rates of the hydrogen and air to the FID were both adjusted to 270 mL/min. The flow rate of the air supplied as a makeup gas was 30 mL/min. The air flow rate to the ozone generator was 60 mL/min. The fused-silica capillary column used was DB-1 (30-m length, 0.25-mm i.d., and 0.25-μm thickness; J&W Scientific, Folsom, CA). The oven temperature was programmed from 200 to 300 °C with a rate of 35 °C/min. The temperatures at injection port and detector were both 330 °C. The splitless injection mode was used for 0.25 min after the injection. The pressure in the injection port with helium as a carrier gas was kept at 60 psi immediately after the injection and then reduced to 30 psi at the deceleration speed of 99 psi/min.

A Varian (San Fernando, CA) model SPS 24 Vac Elut, an Iwaki (Tokyo, Japan) model V-S shaker, and a Kubota (Tokyo, Japan) model KS-5000 centrifuge were used to extract the drug and metabolites from plasma. Two evaporators to remove solvents, a Tokyo Rika Kikai (Tokyo, Japan) model TVE 2000 and a TAITEC (Tokyo, Japan) model TAH-2G (Dry Thermo unit), were used under reduced and normal pressures, respectively.

Materials. Hydrogen and helium gases and compressed air were purchased from Nihon Sanso (Kawasaki, Japan); Bond Elut Certify II columns (3 mL, 200 mg) were purchased from Varian. Two kinds of tubes, 10-mL conical tube and 12-mL centrifuge tube, were used. Both Pyrex tubes were silanized with bis(trimethylsilyl)trifluoroacetamide (BSTFA). A disk, which was made from an alloy of platinum and gold (60:40) and originally shaped like a hat for use as a spinning nozzle in chemical fiber industries, was cut into a plate to have the same diameter as the HP original gilded gas seal (on brass) and a small hole of ~0.6-mm diameter at the center. The diameter and the thickness of the disk were 9.85 and 0.5 mm, respectively. This disk was purchased from Kasen Nozzle Manufacturing Co., Ltd. (Osaka, Japan). The disk was placed in duplicate on the original gas seal.

Chemicals. S-1452 is a dihydrate monocalcium salt of (1*R*,2*S*,3*S*,4*S*)-(5*Z*)-7-(3-((phenylsulfonyl)amino)bicyclo[2.2.1]hept-2-yl)hept-5-enoic acid, (+)-S-145 (**I**). The structures of **I**, its metabolites, and internal standards (IS-I, IS-II) are shown in Figure 1, together with the metabolic pathways.[23] These compounds were synthesized in our laboratories. A silylation reagent, BSTFA, containing 1% trimethylchlorosilane was purchased from Pierce (Rockford, IL). Diazomethane solution was prepared as described previously.[24]

Assay Procedure. Method A: Free Forms (Unconjugated Forms) of I and Its Nine Metabolites. Pipet 2.0 mL of plasma sample into a 12-mL centrifuge tube. Add 2.0 mL of acetone, shake the tube by hand two or three times, then add 0.5 mL of IS-I solution (1 μg/mL), and vigorously shake the tube by hand several times. After centrifuging at 2300*g* for 10 min, transfer 1.0 mL of the supernatant solution into a 10-mL conical tube. Add 1 mL of 0.25 M acetate buffer (pH 7.0) to the tube, and then evaporate the acetone to about half the volume under a gentle stream of nitrogen gas on a TAITEC evaporator set at 30 °C. Add a further 3 mL of the same acetate buffer.

Separately, prepare a solid-phase extraction system with the Vac Elut. Install the stopcock of the vacuum manifold and connect the Certify II extraction columns by opening the stopcock. Place 12-mL centrifuge tubes in the collector rack of the Vac Elut. First, set the turntable to the position "Waste". Add 3 mL of methanol to the extraction columns, conduct suction with an aspirator under reduced pressure of 1−3 in.Hg to trap the elute into a waste bottle, and repeat the same procedure. Stop suction to prevent the column from drying when the methanol layer is about 1−2 mm

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from the sorbent tops. Rinse the columns with 5 mL of 0.25 M acetate buffer by conducting suction under reduced pressure at 2−4 in.Hg until the buffer surface comes close to the sorbent tops. Second, apply the treated plasma sample to the conditioned columns and conduct suction under reduced pressure of 1−2 in.Hg until the surface of the sample solution falls to the sorbent tops. Rinse the columns with 2 mL of 0.25 M acetate buffer twice under a reduced pressure of 1−3 in.Hg. Continue suction and drying of the Certify II columns under a pressure of 10 in.Hg for 30 s. Finally, change the position of the turntable to "Collect" to elute analytes in the tube with 5 mL of 0.75% acetic acid in ethyl acetate under a pressure of 1−3 in.Hg.

Add 0.5 mL of water to the tube removed from the Vac Elut, shake for 3 min, and centrifuge at 1200*g* for 3 min. Transfer the entire organic solvent to a 10-mL conical tube and evaporate at room temperature under reduced pressure on a TVE 2000 evaporator. Further evaporate to dryness at 75 °C for 5 min under a gentle stream of nitrogen gas on a TAITEC dry bath. Dissolve the residue with 75 μL of methanol. Add 0.25 mL of the diazomethane solution, and leave the reaction mixture for 10 min at room temperature. Add 10 μL of glacial acetic acid to stop the reaction, and then evaporate the reaction mixture to dryness at 75 °C for 5 min under a gentle stream of nitrogen gas on the dry bath. Dissolve the residue with 100 μL of acetonitrile, and add 50 μL of BSTFA. Stopper the tube tightly with a well-fitted glass stopper, heat at 75 °C for 15 min on the dry bath, and then evaporate to dryness at 75 °C for 5 min under a gentle stream of nitrogen gas. Dissolve the residue with 15−100 μL of toluene. Adjust the toluene volume appropriately to obtain the concentration desired. Inject 1−5 μL of the solution into the CGC−SCD system. Measure the peak height ratio of each analyte to IS-1.

Method B: Total Forms (Free and Conjugated Forms) of I and Its Nine Metabolites. Use the treated solution of plasma sample (acetone solution) of method A. Evaporate a mixture of the supernatant solution (1.0 mL) and 0.25 M acetate buffer (1 mL) to about half the volume at 30 °C under a gentle stream of nitrogen gas. Add 0.4 mL of 5 M NaOH, shake gently by hand, and then allow to stand for 15 min at room temperature. After addition of 0.4 mL of 5 M HCl and 3 mL of 0.25 M acetate buffer, shake gently by hand. Adjust the pH to 7.0 ± 0.5 with 1 M NaOH and/or 1 M HCl using a pH test paper. Use the sample solution for solid-phase extraction with Vac Elut and proceed as directed in method A.

Standard Solution. Dissolve **I** and its nine metabolites in methanol and then dilute with water to prepare three levels of the stock solutions: 0.05 or 0.1, 0.5 or 1, and 5 or 10 μg/mL. Analysis of compound **VI** was not done because it had not been found in human plasma in a preliminary test.

Calibration Graphs. Place 1.0 mL each of human control plasma into five 12-mL centrifuge tubes. Add 1.0 mL each of acetone to the tubes, and shake them by hand two or three times. Then add the required volume (μL) of the stock solutions and 0.25 mL each of the IS-I solution (1 μg/mL) to the tubes to prepare five concentration levels of plasma standard solutions of 0.5−500 ng/mL for **I** and **IX**, 1−250 ng/mL for **II**−**IV**, **VII**, and **VIII**, and 1−500 ng/mL for **V**, **X**, and **XI**. Shake the tubes vigorously by hand several times. Centrifuge them at 2300*g* for 10 min. Transfer 1.0 mL each of the supernatant solutions into five 10-mL conical tubes. Add 1 mL each of 0.25 M acetate buffer to the tubes and proceed as directed in the procedure.

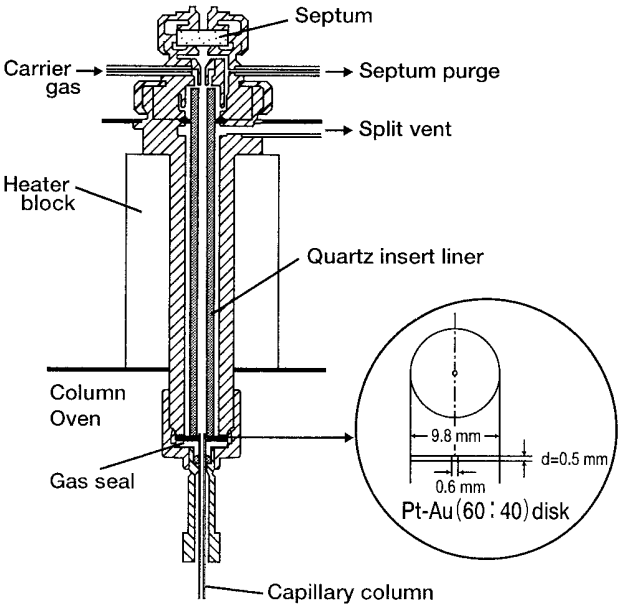

Figure 2. Injection port and Pt−Au (60:40) disk on a gas seal.

RESULTS AND DISCUSSION

Oxidation of the Gas Seal at the Injection Port. The injection port in the cylindrical stainless steel body of the HP gas chromatograph is shown in Figure 2.

The inner part consists of a quartz insert liner and a gas seal at the bottom. Sample is injected to the injection port heated at 330 °C. The vaporized sample is conducted to the capillary column through the small orifice in the gas seal. When the gaseous analyte chemical species came into contact with the inside quartz wall and the gas seal, it caused the gloss on the gold surface to disappear after several heatings of the injection port in the analytical procedure. Furthermore, pinholes formed on the gold surface, and the ground brass became discolored and appeared to be corroded. Repeated working of ~200 analyses caused the surface color to blacken and the number of pinholes to increase. Such changes of appearance suggested that metal oxidation with the sample gases had occurred at the injection port.

Under these conditions, HP gas chromatography was conducted for five compounds (**I**, **V**, **VI**, **IX**, and IS-II). The first analysis yielded five sharp peaks with satisfactory resolution. The peaks of compounds **V** and **VI** were separated at the baseline, although their retention times were very close. The resolution as the *R* value between the two compounds was 0.97. However, the peak heights of the two compounds were reversed and separation worsened with several analyses: the baseline separation of the two peaks became incomplete with the resolution falling to $R = 0.89$. At ~200 analyses, all of the peaks broadened to have very small peak heights, and the resolution between **V** and **VI** fell to $R = 0.43$.

Such band broadening of the peak and insufficient reproducibility of the peak output have been presumed to be caused by the adsorption and/or loss of the sulfur-containing compounds on the inner surface of the flow line.[3,4,25] We assumed that these

phenomena were also induced by the interaction and/or reaction of the sulfur-containing compounds coming into contact with the oxidized metal gas seal in the injection port. The expanded diagram of Figure 2 shows a disk (9.8-mm diameter and 0.5-mm thickness) made from an alloy of platinum and gold (60:40) resistant to heat and oxidation. The alloy has a moderate hardness and maintains its shape under repeated heat expansion. With the disk mounted in duplicate on the gas seal in the injection port, the seal remained stable under the chromatographic conditions and no change occurred on the surface of the disk. When analyses were conducted by use of the disk, the peak form and resolution were reproducible and baseline separation between **V** and **VI** was maintained. The output peaks of the five compounds maintained their resolution even at ~1000 analyses. These facts imply that no interaction occurs even if sulfur-containing analytes come into contact with the surface of the oxidation-resistant alloy.

Improvement of Method for Eliminating Ozone. In the commercial CGC−SCD system, excess ozone after the chemiluminescence reaction is trapped with hopcalite using a vacuum pump. Hopcalite, as the oxidation catalyst[21] in the trap, oxidizes ozone to produce oxygen. Also, corrosive gases such as oxides of nitrogen from the analyte are further oxidized to inert products[1,3] and removed by the pump. However, the reaction of ozone with the catalyst produced fine particles by continuous draft to the trap. The production of the particles caused the following problems for the analysis. First, the fine particles were mostly sent to the exhaust by the vacuum pump, but some moved toward the chemiluminescence reaction cell to adhere to the surface of the UV filter for wavelength selection. The resulting contamination lowered the emission intensity. Second, as the particles sucked into the pump increased, the pressure of 10−13 mmHg, which was required for ozone generating, dropped to 20−30 mmHg.

We noticed that ozone can be changed to water by reduction with a nickel catalyst at room temperature.[22] So, we attempted to replace the hopcalite with a pellet-shaped nickel catalyst. This led to efficient production of water when ozone came into contact with the pellets in the trap. These pellets are processed with a high degree of compressive strength (150 kg/cm^2) and can be used for a long period of time without being crushed. Their use eliminated the problem of contamination of the optical filter by fine particles, and the SCD measurements could be done for 4−5 h a day over 4−5 months. Water produced by the combustion in FID and by the reaction of ozone with the nickel catalyst was sent to the vacuum pump where it formed a water−oil suspension. To eliminate water from the oil and to maintain the vacuum level in the SCD cell, an oil filtration system was connected to the pump. The separated water was easily removed from the upper layer of the oil through its circulation between the pump and the oil filtration system. The vacuum in the SCD cell could be maintained at the proper pressure of 10−13 mmHg even after the system had been used for a long period.

Effect of Purity of Air on Background Noise. The purity of the air to generate ozone significantly affected the background signal. The background signal increased with the increase of SO_2 content in the commercially available compressed air, "pure air B" and "pure air S". In pure air B, the contaminant SO_2 level was less than 0.1 ppm and methane was less than 1.0 ppm. "Pure air S", which is used as a standard gas for analysis of sulfur components in environmental air, contains less than 0.01 ppm of SO_2 and less than 0.1 ppm of methane. The noise level decreased to less than half of that detected with pure air B. The background signal was almost constant with various amounts of hydrogen and air, which were used at 230−300 mL/min.

Optimization of the Amounts of Hydrogen and Air for SCD. Production of sulfur monoxide in a hydrogen-rich air flame affects the sensitivity and stability of the chemiluminescence.[1,2,4,25,26] The response of the flame SCD appears to have a local maximum at certain hydrogen/air ratios and optimization of the response is more difficult than for the flameless SCD.[27] The yield of sulfur monoxide should be raised as much as possible and it should be prevented from being transformed to sulfur dioxide under the following concomitant equilibrium:

$$SO_2 + H_2 \rightleftarrows SO + H_2O \qquad (1)$$

To obtain a higher response in SCD, the amounts of gases and their ratio were examined systematically by flowing air at 200−410 mL/min and hydrogen at 170−380 mL/min, using compound **I** as a sample. Increasing the amount of hydrogen under a constant volume of air at 275 mL/min led to the maximum response when both gases were 1:1. Above 300 mL/min, the response decreased due to the drop in flame temperature. When the flow rates of both gases at a 1:1 ratio were tested in the range of 200−310 mL/min, the maximum peak area was obtained at 270 mL/min.

Specificity of Determination of Biological Samples by CGC−SCD. The advantage of SCD for specificity of sulfur detection in complex matrixes was confirmed by comparison with nitrogen/phosphorus detection (NPD) of these compounds.[28] The CGC−NPD chromatogram of the plasma blank gave many peaks on a large broad peak with a decreasing tailed shoulder, indicating a large amount of biological materials being detected. On the other hand, no interfering peaks were observed in the CGC−SCD chromatograms of the plasma blank and sample. The selectivity factor of sulfur in SCD was calculated to be more than 10^7, much higher than that of nitrogen in NPD. In CGC−SCD, little or no change in the baseline such as a drifting upward and unevenness was observed even when the injection volume was increased under high pressure (pulsed splitless injection) or the analysis was done with increasing temperature.

Drug and Metabolite Analysis. The CGC−SCD method was applied to determine the free form of **I** and its nine metabolites in human plasma. They are all carboxylic acids, which exist as both the free form and the glucuronide. Previously, we analyzed **I** and its metabolites in human urine using a capillary gas chromatograph connected to a sulfur-specific Hall detector system (ELCD),[24] which measures the electrolytic conductivity for SO_2 or SO_3 produced after the combustion of sulfur-containing compounds. However, the sensitivity of the ELCD was too low to detect **I** and its metabolites at the nanogram order of concentration in human plasma. In contrast, the CGC−SCD method was

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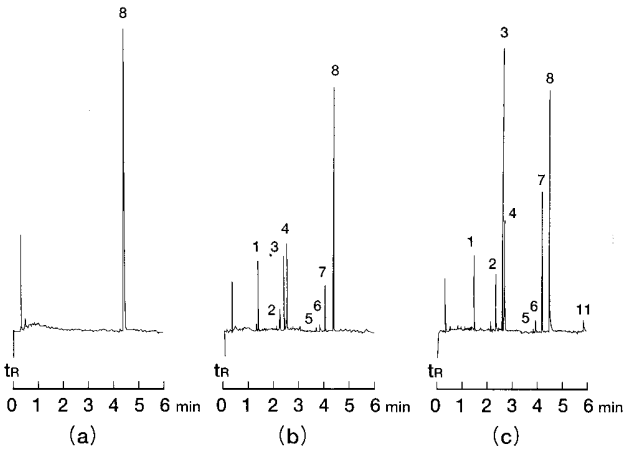


Figure 3. Typical capillary gas chromatograms of (+)-S-145 and its metabolites. (a) Background for human control plasma. (b) Method A treatment of plasma sample collected 1 h after single oral administration of 50 mg of S-1452. Peak (concentration, ng/mL): 1, **IX** (68.2); 2, **X** (27.4); 3, **XI** (107.2); 4, **V** (132.5); 5, **VII** (7.7); 6, **VIII** (8.9); 7, **I** (76.1); 8, IS-I. (c) Method B treatment of plasma sample collected 1 h after single oral administration of 50 mg of S-1452. Peak (concentration, ng/mL): 1, **IX** (98.0); 2, **X** (97.0); 3, **XI** (454.9); 4, **V** (152.9); 5, **VII** (10.7); 6, **VIII** (13.5); 7, **I** (275.9); 8, IS-I; 11, **IV** (18.2).

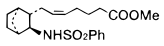
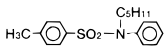
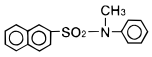
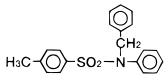
sensitive enough to determine the plasma level, which is 50−100 times lower than the concentration in the urine.

The glucuronides of **I** and its metabolites were hydrolyzed to their free forms in alkaline solution before the Bond Elut Certify II treatment (method B). After the pH adjustment, the free forms were analyzed in the same manner as directed in method A. The amount of glucuronide was calculated by subtracting that of the free form (method A) from the total amounts of the glucuronide and free form (method B). Parts b and c of Figure 3 show the two chromatograms obtained by treating the human plasma, which was collected 1 h after oral administration of 50 mg of S-1452, according to methods A and B, respectively.

Figure 3c shows a significant increasing amount of **X**, **XI**, **I**, and **IV** in comparison with Figure 3b, indicative of their glucuronide formation. No interfering peaks were found from the control plasma collected before administration, as shown in Figure 3a.

Method Validation. Standard solutions containing 11 compounds (analyte and the internal standard) spiked with human plasma were prepared. Replicate measurements of the compounds showed good reproducibility of the retention times with relative standard deviations at the 0.1% level. Ten compounds in five standard solutions, ranging in concentration from 0.5 or 1 to 250 or 500 ng/mL, were simultaneously analyzed by the procedure. The peak height ratio of each compound to the internal standard against the concentration gave good linearity with the correlation coefficient more than 0.999. The precision of the method was estimated using the three standard solutions at minimum (0.5 or 1 ng/mL), medium (125 or 250 ng/mL) and maximum (250 or 500 ng/mL) levels. Ten compounds in these solutions were simultaneously assayed 6 times a day. The relative standard deviations at the medium and the maximum level concentrations for each compound were within 10%. The variation increased significantly at the minimum concentration level, but did not exceed more than 20% for each compound. The assay was repeated for 3 days. There was no tendency toward significant variation among the 3 days. The lower quantitaion limits were 0.5 ng/mL for **I** and **IX** and 1 ng/mL for the other compounds.

Table 1. Relative Response Factor of Sulfonamide Compounds

	Compound	Relative response factor
1.		1.00
2.		1.03
3.		1.18
4.		1.12

Applicability of CGC−SCD to Analysis of Other Sulfur-Containing Compounds. The method was also applied to the analysis of four other sulfur-containing compounds described in Table 1. The gas chromatographic conditions used above were modified by appropriately changing the temperature, column, column head pressure, etc. As shown in Table 1, the relative chemiluminescence response factors for these compounds were in the range of 1.00−1.18, indicating a stoichiometric response for sulfur in the molecule. The compounds spiked with guinea pig plasma were assayed to construct the calibration graphs. The obtained output responses were all linear in the range from 1 to 500 ng/mL with the correlation coefficients more than 0.999.

Received for review August 17, 1999. Accepted November 8, 1999.

AC990927L