Determination of Acetylsalicylic Acid and Salicylic Acid in Plasma

By MALCOLM ROWLAND and SIDNEY RIEGELMAN

A specific and sensitive method has been developed for the determination of acetylsalicylic acid and salicylic acid in plasma. Both of these compounds are extracted from acidified plasma with ether. A portion of the ether extract is concentrated, reacted with a silanizing reagent in carbon disulfide, and the silyl derivative of acetylsalicylic acid is analyzed by gas–liquid chromatography using dibutyl maleate as the internal standard. Another portion of the ether extract is extracted with phosphate buffer and the salicylate read spectrophotofluorometrically. Only 0.5 ml. or less of plasma is required for analysis and one can readily determine 0.4 mcg. acetylsalicylic acid/ml. in the presence of 100 mcg. salicylic acid/ml.

IN THE PAST, whole blood and plasma acetylsalicylic acid (ASA) levels have been calculated as the difference in the salicylic acid (SA) concentrations before and after hydrolysis of an aqueous extract of the sample (1–3). Such methods have large errors when SA is present in vast excess of ASA, a situation which often occurs in practice. Recently, ASA has been separated from SA by a paper chromatographic method (4) or by selective extraction (5), and assayed spectrophotofluorometrically after subsequent hydrolysis of ASA to SA.

Direct determinations of ASA have been made using gas–liquid chromatography (GLC) (6, 7) but these have been confined to tablet formulations. Crippen *et al.* (8) have chromatographed ASA as its methyl ester, while Horii *et al.* (9) have separated a large number of aromatic acids, including SA, as their trimethylsilyl derivatives. However, in none of the above methods has ASA been determined specifically in blood or plasma. A specific and sensitive method for the determination of ASA and SA in plasma, requiring only 0.5 ml. sample, is now presented. The ASA is determined directly by GLC as its trimethylsilyl derivative and the SA is determined spectrophotofluorometrically.

EXPERIMENTAL

Materials—The majority of materials used were analytical grade from Baker Chemical Co. The ASA sample from Merck contained less than 0.1% SA as shown by spectrophotofluorometric assay. Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), and most gas chromatographic materials were obtained from Applied Science Laboratories.

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Apparatus—An F & M model 700 gas chromatograph, with a flame ionization detector, model 810 electrometer, 220 power proportioning temperature controller, and a Hewlett-Packard 7127 A recorder, was used for ASA determinations. An Aminco-Bowman spectrophotofluorometer, model 8210, was used for SA assays.

Analytical Procedure

Reagents—(*a*) Fifty per cent w/v potassium fluoride in distilled water. (*b*) Heparin solution 1000 units /ml. (free of preservatives). (*c*) Distilled ether, prepared daily. (*d*) Distilled carbon disulfide, prepared daily. (*e*) Five per cent w/v potassium hydrogen sulfate in distilled water. (*f*) Hexamethyldisilazane (HMDS). (*g*) Trimethylchlorosilane (TMCS). (*h*) Dibutyl maleate standard solution, 1 mcg./ml. ether. (*i*) Carbon disulfide solution; each ml. CS_2 containing 2 μl. silanizing reagent (20 parts HMDS/1 part TMCS). (*j*) Phosphate buffer (0.1 *M*), pH = 7.0.

Collection and Storage of Samples—A 5-ml. sample of whole blood was placed immediately into a centrifuge tube containing 50 μl. heparin solution and 50 μl. potassium fluoride solution, the tube being kept in an ice bath. The blood was gently mixed and kept in the ice bath for 2 min. (final blood temperature = +2°). The blood was then centrifuged, and the plasma pipetted off and stored in dry ice until required for analysis.

Analysis—Plasma (0.05–0.5 ml.) was pipeted into a centrifuge tube containing 0.5 ml. potassium bisulfate solution (which acted as an acidifying medium and adjusted the pH to 2.0), extracted with 7 ml. of ether, and then centrifuged.

ASA Assay—A 5-ml. aliquot of the above ethereal extract was pipetted into a centrifuge tube containing 1 ml. dibutyl maleate standard (1 mcg./ml. ether). The combined ethereal solutions were concentrated on a water bath at 42° to about 50 μl. Carbon disulfide reagent (1 ml.) was then added to the solution which was then once again concentrated on a water bath at 60° to about 50 μl. Approximately 1 μl. of the concentrate was injected for analysis into the chromatograph. The peak height ratio of ASA derivative to dibutyl maleate was measured and the concentration of ASA determined by reference to a calibration curve. Freshly prepared ASA aqueous standards (1–10 mcg./ml.) were assayed in the same manner and run with each batch of plasma samples.

The column was 100–120 mesh Gas Chrome Q onto which was coated 3% DC QF-1, and packed into a 7-ft., 1/8-in. o.d. stainless steel tube. The op-

erating conditions were as follows: column temperature, 125°; injection block and detector temperatures, 200° and 180°, respectively; nitrogen pressure, 40 lb./sq. in.; hydrogen pressure, 16 lb./sq. in.; air pressure, 20 lb./sq. in.

SA Assay—A 1-ml. aliquot of the above ethereal extract of the acidified plasma was re-extracted with 5 ml. phosphate buffer (pH 7.0) in a centrifuge tube. The sample was centrifuged, excess ether sucked off, and nitrogen blown through the phosphate buffer for 30 sec. to remove the dissolved ether (which modified the spectrophotofluorometric readings). The solutions were then read on the spectrophotofluorometer, activation wavelength, 315 mμ. (uncorrected); emission wavelength, 420 mμ (uncorrected).[1] Fluorescent measurements of standard solutions of SA in phosphate buffer (0.1–1.0 mcg./ml.) were made at the same time. In a number of samples the phosphate solutions were heated for 1 hr. at 100° to hydrolyze the ASA to SA, and the total SA was then measured.

Calibration Curves—The following concentrations of ASA and SA were added to plasma obtained from a subject who had not taken any drugs during the previous week (mcg. ASA/ml.–mcg. SA/ml.: 0/0, 0.4/100, 1.0/80, 2.0/40, 4.0/20, 10/10, and 20/4.0). The samples of plasma were then assayed for ASA and SA by the above methods. With ASA, the peak height ratio of ASA to dibutyl maleate was plotted against the concentration of ASA; and with SA, the fluorescence was plotted against the concentration of SA.

RESULTS AND DISCUSSION

Inhibition of the Hydrolysis of ASA in Whole Blood—The hydrolysis of ASA in whole blood at 37° is very rapid, $t_{1/2}$ = 30 min. (10). Therefore, it is very important to inhibit the enzymatic hydrolysis after withdrawal of blood samples; otherwise, appreciable error in the estimation of ASA occurs. Normally the hydrolysis has been stopped by addition of whole blood into acid. However, this also hemolyzes the red blood cells and this was to be avoided since the interest was in assaying ASA plasma levels.

Initially, the concentration of free[2] and total[2] SA was determined, by the method of Harris and Riegelman (10), in blood samples taken at 2, 5, 10, and 15 min. after the addition of 1 ml. of solution containing 200 mcg. ASA/ml. of normal saline to 9 ml. of freshly drawn heparinized blood. The blood was left out at room temperature. The above experiment was repeated with the addition of 5 mg. potassium fluoride/ml. blood (100 μl. of 50% KF per 10 ml. blood) prior to mixing with the ASA solution. No hemolysis of the blood occurred when using this amount of potassium fluoride. The hydrolysis of ASA was also determined when blood was maintained in an ice bath (+1°) both with and without the addition of 5 mg. KF/ml. blood. The appearance of free SA, rather than the disappearance of unchanged drug, was used to calculate the degree of hydrolysis. The results, given in Table I, indicate that while fluoride and cold each decrease hydrolysis, the combination of both is best.

Table I—The Effect of Temperature and Potassium Fluoride on the Hydrolysis of Acetylsalicylic Acid in Whole Blood

Conditions	% ASA Hydrolyzed					
Time, min.:	2	5	10	15	20	60
Room temp.	3.5	9.6	13.6	21	...	...
Room temp. + KF (5 mg./ml.)	1.5	1.8	7.0	11	...	...
Ice bath (+1°)	...	3.7	...	8.6	...	...
Ice bath (+1°) + KF (5 mg./ml.)	...	0.8	0.8	...	0.85	1.1

In a separate experiment, the effect of centrifugation on the hydrolysis of ASA was investigated. Fresh heparinized blood containing 20 mcg. ASA/ml. and 5 mg. KF/ml. was cooled in an ice bath and divided into four parts. Each sample of whole blood was immediately assayed for free and total SA and the remainder of each sample was centrifuged for 3 min., the buckets of the centrifuge being previously cooled in a refrigerator. Each centrifuged sample was then shaken and assayed for free and total SA. It was found that there was no increase in the degree of hydrolysis of ASA when centrifuged under these conditions. Therefore, since the total time taken between withdrawal of the blood sample and the separation of the plasma is approximately 15 min., the hydrolysis of ASA was never greater than 2%.

Preparation of Sample for Analysis—Potassium hydrogen sulfate is a useful acidifying agent; a 5% solution has a pH of 1.6. The mixing of an equal volume of this solution with plasma gives a final pH of 1.9–2.2. Under these conditions extraction of ASA and SA with ether is quantitative, as shown by spectrophotofluorescent measurements.

Gas Chromatography—Marked difficulty was experienced in chromatographing free ASA or SA on isophthalic acid/Carbowax terephthalic acid and terephthalic acid/FFAP columns. In all cases a complex peak was obtained with both SA and ASA, while a symmetrical peak was obtained with benzoic acid. This phenomenon may be due to the decarboxylation of SA and ASA at the operating temperatures of 180° or more required for these columns.

A symmetrical peak of the ASA-trimethylsilyl derivative was obtained on SE-30, QF-1, and XE-60 columns. Although three derivatives of salicylic acid are possible with HMDS, only two derivative peaks were obtained, a major and a minor one. These were not investigated further, other than it was noted that they were obtained in the same ratio after repeated recrystallization of SA. The QF-1 column gave the best separation of the ASA and SA derivative peaks and thus was chosen for the analysis of ASA.

Carbon disulfide was chosen as the final reaction solvent since the flame ionization detector does not respond to this compound. The formation of the TMS derivative appeared to be almost spontaneous and quantitative in this solvent, since there was no increase in the peak height ratio of ASA–TMS derivative to internal standard before or after heating the reaction mixture for 1 hr. at 40°.

[1] Although ASA is extracted into the phosphate buffer, this compound does not fluoresce. Neither does ASA quench the fluorescence of SA since varying amounts of ASA did not affect the linearity of the SA calibration curve. However, it is important to read the SA fluorescence promptly since ASA does hydrolyze in phosphate buffer ($t_{1/2}$ at 25° approximately 2.5 days) liberating SA.

[2] The term free SA is used here to mean both protein bound and unbound SA, and total SA is the sum of the free SA and that produced by the hydrolysis of ASA to SA.

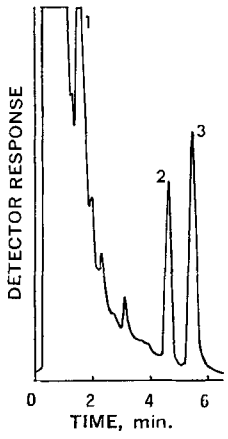

Fig. 1—Chromatogram of an extract of plasma from a subject who had taken an oral dose of acetylsalicylic acid. Key: 1, salicylic acid–trimethylsilyl derivative (T_r = 1.5 min.); 2, dibutyl maleate (marker; T_r = 4.6 min.); 3, acetylsalicylic acid trimethylsilyl derivative (T_r = 5.4 min.).

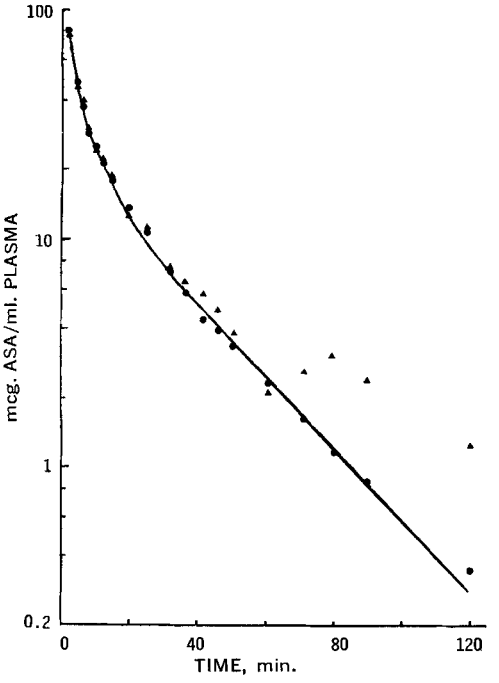

Fig. 2—Comparison of the gas–liquid chromatographic method (●) with the differential salicylic acid method (▲) for the determination of the acetylsalicylic acid plasma levels after the intravenous administration of acetylsalicylic acid.

A large number of compounds were screened as an internal marker. The most promising were methyl myristate, methyl tridecanoate, dimethyl phthalate, and dibutyl maleate; the last proved the best. Dibutyl maleate is soluble and stable in ether and carbon disulfide, does not react with HMDS, and gives a symmetrical peak close to the ASA–TMS derivative (Fig. 1).

Determination of SA in Plasma—Although SA gives a TMS derivative with HMDS, the drug was assayed spectrophotofluorometrically. The reasons for this were: (*a*) the retention time for the major SA–TMS peak was 1.5 min., which is on the solvent peak, rendering measurements of this peak height extremely difficult and inaccurate with concentrations of less than 10 mcg./ml. (*b*) Since generally SA concentrations are much larger than the ASA concentrations, constant changing of the attenuation switch would be necessary if both the SA and the ASA peaks are to be seen on the same scale. This demands constant attention of the analyst. On the other hand, the fluorescence method is sensitive and all samples can be analyzed in one batch.

Specificity, Sensitivity, and Reproducibility—Plasma from many subjects who had not ingested either ASA or SA were analyzed for these drugs. The GLC method for ASA was found to be specific, as no peak was ever observed, in these blank plasma samples, at the same retention time as the ASA–TMS derivative or that of the internal marker, dibutyl maleate. Also, neither salicylamide, which occasionally is formulated with ASA, nor the metabolite of SA, salicyluric acid, gave interfering peaks under the present assay conditions. The GLC method is quite sensitive as 0.2 mcg. ASA/ml. plasma could be readily demonstrated. The fluorescent method for SA determination is also very sensitive, and it is reasonably specific as no blank plasma sample gave a fluorescent reading greater than that equivalent to 0.5 mcg. SA/ml. plasma.

For reproducible results in the ASA determinations it was found necessary to saturate the column. This is readily achieved by rapidly injecting 4–5 samples and waiting about 15 min. for the column to clear. When this was carried out, the peak height ratio of standards were within 2% at the beginning and end of the run, and the calibration curve was linear from 0.4–20 mcg. ASA/ml. plasma. Also, samples of plasma with ASA added in concentrations of 1.0, 10, and 20 mcg./ml. were analyzed for ASA. (Seven samples were analyzed at each concentration.) The relative standard deviations (in per cent) of peak height ratio at 1, 10, and 20 mcg. ASA/ml. plasma were 1.8, 2, and 1.5%, respectively.

The SA calibration curve was linear from 2–100 mcg. SA/ml. plasma and the relative standard deviations (per cent) of the fluorescent readings at 5, 20, and 50 mcg. SA/ml. plasma were 1.7, 2.1, and 0.8%, respectively. (Seven samples were analyzed at each concentration.)

In addition, it was shown that both the ASA and SA calibration curves were unaffected by the presence of a large amount of the other drug, *e.g.*, with 0.4 mcg. ASA/ml. plasma the same peak height ratio was obtained in the presence or absence of 100 mcg. SA/ml. plasma. This further demonstrates the specificity of each of these assays.

Stability of ASA and SA in Plasma—Plasma samples, obtained from subjects who had taken ASA, were analyzed for ASA and SA. Analyses were made immediately, and 1, 2, 3, and 7 days after collection of the samples, and then once again after 30 days. All plasma samples were stored at dry ice temperature.

No loss of ASA or SA occurred in any samples stored up to 1 week. Further, several samples have been studied after 1 month or more of storage, and the variations of results were within the standard error of the method.

CONCLUSIONS

The present method for the simultaneous determinations of ASA and SA in plasma is simple and relatively rapid, since at least 20 samples can be analyzed in 5 hr. The stability of ASA under the sample and storage conditions enable determinations to be made at the analyst's convenience. The advantage of this method over the differential SA method for the determination of low concentrations of ASA in the presence of large amounts of SA is clearly shown in Fig. 2. Forty minutes after the administration of 650 mg. of ASA, the SA levels usually reach 40–50 mcg./ml., and shortly thereafter one must be able to determine as little as 0.4 mcg. ASA/ml. in the presence of the above vast excess of SA. It is only with a GLC method that the biological half-life of ASA can be accurately determined. Also, the recently proposed alternative method of Cotty *et al.* (5) may not be any more useful than the differential methods due to relative errors indicated in the published results. The methods presented in this paper are now being used to study the pharmacokinetics of ASA and SA in man and to investigate the influence of dosage forms on the absorption of ASA.

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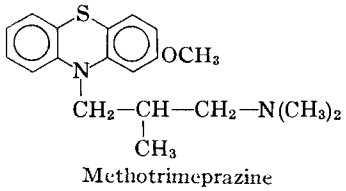
Estimation of Methotrimeprazine in Brain and Correlation of Brain Levels with Pharmacologic Activity

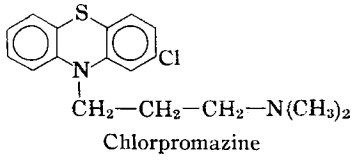
By ABDEL-HALIM M. AFIFI* and E. LEONG WAY

A method for estimating methotrimeprazine in brain by ultraviolet spectrophotometry was developed which is based on extracting the compound from alkalinized tissue with 1 per cent butanol in cyclohexane and determining its absorbance in acid at 250 mμ. A high degree of specificity for methotrimeprazine was conveyed to the procedure by subjecting the organic solvent extract of tissue to several buffer washes. Following intravenous injection of the compound in mice, methotrimeprazine was found to be metabolized rapidly during the first hour, after which its rate of disappearance slowed considerably. Brain levels were maximal at 1 hr. and were still appreciable at 4 hr. The onset and duration of pharmacologic effects as measured by a loss in coordinated motor ability varied directly with the concentration of drug in the brain, suggesting that the parent compound is principally responsible for mediating the response.

THE PHENOTHIAZINE DERIVATIVE, methotrimeprazine maleate (levomepromazine, and R.P. 7044),[1] until recently an investigational drug in the United States,[2] has been used for some time in Europe by psychiatrists as an alternative for chlorpromazine. The structures of the two agents are shown for comparison.



Methotrimeprazine



Chlorpromazine

The pharmacology of the compound has been studied extensively by Courvoisier *et al.* (1) who reported that its actions are similar to those of chlorpromazine but it is more potent than the latter with respect to sedative and hypothermic properties and capability of potentiating the

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[1] Marketed as Nozinan by Specia, Paris, France, and Veractil by May & Baker, Dagenham, Essex, England.
[2] Since the writing of this paper, this drug has been marketed in the U.S. as Levoprom by Lederle Laboratories, Pearl River, N.Y.