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The estimation of acetylsalicylic acid and salicylate in biological fluids by gas-liquid chromatography

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A gas-liquid chromatographic method is described for the simultaneous separation and determination of acetylsalicylic acid (ASA) and salicylic acid (SA) in biological fluids. The salicylates are completely extracted from deproteinized plasma or urine at pH 2 with ether containing *p*-toluic acid as the internal standard. The silylated derivatives are formed using bis(trimethylsilyl) trifluoroacetamide and separated at 150° on preconditioned 100–120 mesh Gas-Chrom Q coated with 5% OV17 packed into a 6 ft, $\frac{1}{4}$ inch o.d. glass column in a gas chromatograph with a flame ionization detector and integrator. Detector response is linear over the range from 0–2 mg ml^{-1} for SA and from 0–100 μg ml^{-1} for ASA. The time required for the analysis of SA alone or with ASA is about 80 min, the analysis of ASA alone requires about 20 min. The precision of the method is 1% or better for drug concentrations above 10 μg ml^{-1}. The limits of detectability for SA and ASA are 1 and 2 μg ml, respectively.

The relatively frequent occurrence of salicylate poisoning has led to the development of many methods for the estimation of salicylates in plasma and urine, including colorimetric (Brodie, Udenfriend & Coburn, 1944; Smith, Gleason & others, 1946; Trinder, 1954), fluorometric (Schachter & Manis, 1958; Lange & Bell, 1966; Harris & Riegelman, 1967), spectrophotometric (Routh, Shane & others, 1967), thin-layer chromatographic (Khemani & French, 1969) and gas chromatographic (Rowland & Riegelman, 1967) procedures. Gas chromatography is the technique of choice because of sensitivity and specificity. In the method of Rowland & Riegelman (1967) acetylsalicylic acid (ASA) is determined by gas chromatography and salicylic acid (SA) fluorometrically. Studies on the interaction of salicylates with other drugs underway in our laboratory (Coldwell & Thomas, 1971; Thomas, Coldwell & others, 1972) required a procedure for the simultaneous estimation of ASA and SA in blood. The method developed is now described.

ANALYTICAL PROCEDURE

Reagents. Analytical grade SA and ASA; U.S.P. grade sodium salicylate (NASA). Bis(trimethylsilyl) trifluoroacetamide (Regisil; Regis Chemical Company, Chicago), gas chromatographic materials (Applied Science Laboratories). All other chemicals are analytical grade. Ether redistilled in an all-glass unit before use.

Apparatus. A Varian model 2100 or a Perkin Elmer model 900 gas chromatograph with flame ionization detector and Hewlett-Packard model 3370 A integrator.

Column: 100–120 mesh Gas-Chrom Q coated with 5% OV17 packed into a 6 ft, ¼-inch o.d. glass tube and conditioned at 280° for 4 days before use. Operating conditions: column temperature—150°, injection port temperature—200°, detector temperature—250°; flow rates of nitrogen, hydrogen and air: 50, 30 and 300 ml min^{-1}, respectively.

Analysis. Plasma from whole blood is stored as described by Rowland & Riegelman (1967) until analysed. An aliquot (0·05–0·5 ml) of plasma is accurately pipetted into a 12 ml glass stoppered centrifuge tube containing 0·5 ml potassium bisulphate solution (5% w/v $KHSO_4$ in distilled water) and the mixture saturated with solid sodium chloride. Glass-distilled ether, 5 ml, containing 5 μg ml^{-1} *p*-toluic acid as an internal standard is added to the tube and the contents vigorously shaken for at least 3 min. After centrifugation for 10 min at 3000–5000 rev min^{-1}, the upper ether layer is carefully transferred to a 5 ml glass-stoppered centrifuge tube. The ether is evaporated under nitrogen to complete dryness, 40 μl of silylating agent (Regisil) is added under nitrogen and the stoppered tube is placed for 1 h in a water bath at 50°. On removal, the tube is cooled immediately to room temperature (20°) and an aliquot (1–2 μl) of the solution is injected into the gas chromatograph. The retention times of the silylated derivatives of *p*-toluic acid, SA and ASA are 129, 226 and 353 s, respectively.

Standards and calibration curves. Sodium salicylate and ASA standards are prepared by appropriate dilution of aliquots of aqueous solutions containing 20 mg ml^{-1} and 200 μg ml^{-1}, respectively. Known volumes of the dilute solutions are mixed with drug-free plasma (1 : 4) and aliquots taken for analysis as described above. ASA solutions must be freshly prepared and processed immediately. The silylated standards can be stored and reused indefinitely. Typical calibration curves are shown in Fig. 1.

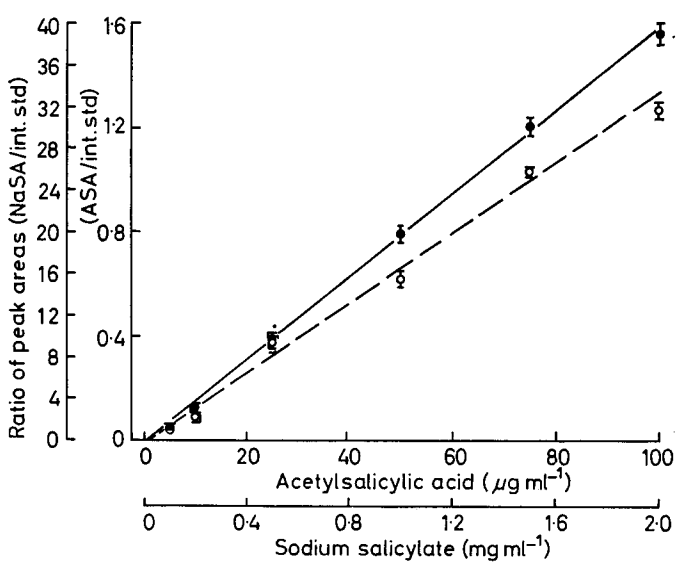

FIG. 1. Calibration curves for sodium salicylate and acetylsalicylic acid.

RESULTS AND DISCUSSION

The extraction of SA and ASA from plasma at pH 2·0 with ether, as described by Rowland & Riegelman (1967), is virtually complete since identical results are obtained when similar concentrations of these salicylates in plasma and in ether are analysed by the described procedure. The silylation step requires 1 h incubation time to ensure complete reaction with SA. ASA, on the other hand, is silylated by Regisil, under the conditions described, almost instantly since the ratio of ASA peak area to internal standard peak area remains constant during the incubation period. Therefore, when measuring only the amount of ASA present the time required for analysis is approximately 20 min.

A typical chromatogram of an extract of plasma taken from a subject 20 min after oral ingestion of 900 mg of ASA is shown in Fig. 2. The concentrations of SA and

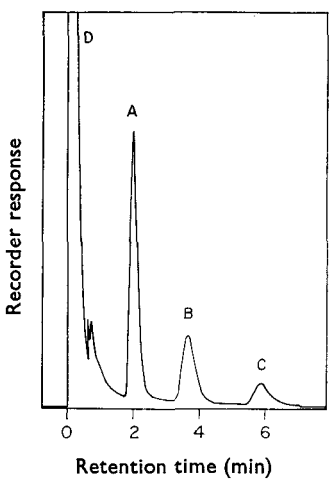

FIG. 2. Gas-liquid chromatographic analysis of plasma for salicylic acid (peak B) and acetylsalicylic acid (peak C). Peaks A and D are the internal standard (*p*-toluic acid) and Regisil, respectively. See text for instrument parameters. The analysis indicated the presence of 16·9 μg ml^{-1} of salicylic acid and 8·3 μg ml^{-1} of acetylsalicylic acid in the plasma 20 min after oral ingestion of 900 mg of acetylsalicylic acid.

ASA, 16·9 and 8·3 μg ml^{-1} respectively, are in the expected range (Cotty & Ederma, 1966; Leonards, 1962). SA concentrations over the range from 0·01 to 2·0 mg ml^{-1} and ASA from 10 to 100 μg ml^{-1} can be estimated with a standard error of $\pm$0·02. Below 10 μg ml^{-1}, the reproducibility of ASA estimations is more variable and the standard error is increased. The accuracy can be improved by starting with a larger sample of plasma.

No difficulty was experienced in estimating ASA in the presence of 10 times the concentration of SA. In a study of the effect of ASA on the metabolism of phenacetin and acetaminophen in man (Thomas, Coldwell & others, 1972), the plasma concentrations of salicylate determined by gas liquid chromatography ranged from 3·1 to 9·3 mg % in 24 volunteers 2 h after the ingestion of 1·36 g of ASA in 3 divided doses over 4 h. These values were in the expected range (Leonards, 1962).

Serum samples taken from hospitalized patients suspected of having ingested salicylates and which had been analysed for salicylate colorimetrically (Keller, 1947), were analysed by the gas-liquid chroamtographic method described above. The results

Table 1. *Colorimetric and gas-liquid chromatographic analysis of serum samples for salicylic acid.* The samples were obtained from patients suspected of salicylate intoxication. Results are expressed in mg %.

	Sample number									
	1	2	3	4	5	6	7	8	9	10
Colorimetric*	69	45	41	42	31	29	23	18	13	12
G.l.c.	70·3	39·5	41·5	38·0	21·5	13·0	9·6	9·9	5·2	4·4

* Keller (1947).

are shown in Table 1. There are appreciable differences, particularly at salicylate concentrations below 40 mg %. Blank readings of 5 mg % are considered normal in Keller's method; also the ferric nitrate reagent is non-specific, forming coloured complexes with any compound having a phenolic hydroxyl group. Barbiturates are less volatile than SA and ASA and have longer retention times (>8 min) under the conditions described and do not interfere with the analysis. Most other commonly used drugs are basic and would not be extracted from plasma with ether at pH 2·0.

REFERENCES

BRODIE, B. B., UDENFRIEND, S. & COBURN, A. F. (1944). *J. Pharmac. exp. Ther.*, **80**, 114–117.

COLDWELL, B. B. & THOMAS, B. H. (1971). *J. Pharm. Pharmac.*, **23**, 226–227.

COTTY, V. F. & EDERMA, H. M. (1966). *J. pharm. Sci.*, **35**, 837–839.

HARRIS, P. A. & RIEGELMAN, S. (1967). *Ibid.*, **56**, 713–716.

KELLER, W. J. (1947). *Am. J. clin. Path.*, **17**, 415–417.

KHEMANI, L. & FRENCH, I. W. (1969). *J. Chromat.*, **41**, 274–275.

LANGE, W. E. & BELL, S. A. (1966). *J. pharm. Sci.*, **55**, 386–389.

LEONARDS, J. R. (1962). *Proc. Soc. exp. Biol. Med.*, **110**, 304–308.

ROUTH, J. I., SHANE, N. A., ARREDONDO, E. G. & PAUL, W. D. (1967). *Clin. Chem.*, **13**, 734–743.

ROWLAND, M. & RIEGELMAN, S. (1967). *J. pharm. Sci.*, **56**, 717–720.

SCHACHTER, D. & MANIS, J. G. (1958). *J. clin. Invest.*, **37**, 800–807.

SMITH, P. K., GLEASON, H. L., STOLL, C. G. & OGORZALEK, S. (1946). *J. Pharmac. exp. Ther.*, **87**, 237–255.

THOMAS, B. H., COLDWELL, B. B., ZEITZ, W. & SOLOMONRAJ, G. (1972). *Clin. Pharmac. Therap.* **13**, 906–910.

TRINDER, P. (1954). *Biochem. J.*, **57**, 301–303.