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Note

Improved gas–liquid chromatographic–flame ionization detection assay of acetylsalicylic and salicylic acids

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Numerous assay methods have been reported for quantitative determination of salicylic acid (SA) and acetylsalicylic acid (ASA) in pharmaceuticals and in biological specimens. Among them, colorimetric[1], UV spectrometric[2,3] and fluorometric[4] methods were commonly used. These techniques are either time consuming or not sensitive. Therefore, a simple method which could detect SA and ASA specifically was necessary. In 1964, a gas–liquid chromatographic (GLC) technique was reported to separate simultaneously and to quantitate ASA and SA in aspirin tablets[5]. Since then, a number of GLC assay papers[6–8] for simultaneous SA and ASA determinations in pharmaceuticals or biological fluids have been reported. However, no one single method was described to quantitate both pharmaceutical and biological media. During an investigation of the applicability of the method by Watson *et al.*[6] for SA and ASA, it was discovered that, with several modifications, the assay method can be used to quantitate pharmaceutical and human plasma samples. Furthermore, the GLC analysis time was shortened approximately five times (from 45 to 8 min). In this paper, this modified and improved GLC method is described.

EXPERIMENTAL

Materials

Salicylic acid (SA), acetylsalicylic acid (ASA), *o*-methoxybenzoic acid, *o*-ethoxyethanol and 5% (w/v) potassium bisulfate were analytical reagent grade (BDH, Poole, Great Britain) N-methyl-N-nitroso-*p*-toluene sulfonamide was purchased from Aldrich (Milwaukee, Wisc., U.S.A.) (Cat. No. D2900-00). Diethyl ether was reagent grade. Methanol, methylene chloride and chloroform (distilled in glass) were supplied by Caledone (Georgetown, Canada).

Gas–liquid chromatography

A Hewlett-Packard Model 5830A gas–liquid chromatograph equipped with a flame ionization detector was used. The GLC conditions for routine analyses were: injection temperature, 180°; column temperature, 145°; detector temperature, 205°; carrier gas (helium) 30 ml/min, using a 1.8 m × 2 mm I.D. glass column packed with 3% OV-17 on Chromosorb W HP (80–100 mesh).

Diazomethane generation and derivative formation

Diazomethane was generated using Levitt's method[9] with slight modifications. The drying compartment was deleted and nitrogen was bubbled directly through a micro-pipette into the generator. Diazomethane, in a gas form, reacted directly with the samples at the receiving end. The appearance of a faint yellow colour in the sample solution indicated the completion of the reaction.

Standard curves

Quantitation of SA and ASA in tablets. Serial dilutions of SA and ASA in chloroform were pipetted into individual 15-ml centrifuge tubes. To the mixtures, 1 ml of *o*-methoxybenzoic acid (10 μg/ml in chloroform) was separately added as internal standard. The contents were dried under a gentle stream of nitrogen. The residue was reconstituted with 100 μl of methylene chloride and derivatized. All samples were kept in ice (4°) until GLC analysis. A 3-μl aliquot of the derivative solution was injected onto the GLC column under the aforementioned conditions. A calibration curve was prepared by plotting the area ratios of either SA or ASA/internal standard against their respective concentrations.

Quantitation of SA and ASA in human plasma. Human plasma (1 ml) samples were spiked individually with serial dilutions of SA and ASA dissolved in water. The samples were adjusted to pH ≈2 with 5% (w/v) potassium bisulfate (final volume 2.5 ml) and shaken with 7 ml of chloroform for 20 min. After centrifugation, 5 ml of the organic phase and 1 ml of internal standard (10 μg/ml chloroform) were transferred to a 15-ml centrifuge tube. The contents were dried under nitrogen, reconstituted in 100 μl of methylene chloride, derivatized and analysed as described. Calibration curves were prepared by plotting the area ratios of SA or ASA/internal standard *versus* the respective concentrations of SA and ASA.

Estimation of ASA and its decomposition product SA in ASA tablets

Ten ASA tablets were weighed accurately and ground in a mortar. An amount equivalent to 300 mg of ASA was weighed accurately and 10 ml of methanol were added to dissolve the SA and ASA content. The resultant suspension was filtered and the filtrate was diluted fifteen times with chloroform for SA determination. This solution was further dileted one hundred times for ASA determination. A 1-ml aliquot of either of the diluted solutions was mixed with 1 ml of internal standard solution. The contents were dried and analysed as described above.

Human study

A healthy subject was fasted overnight prior to the administration of 2 effervescent ASA tablets (650 mg of ASA) in 250 ml of water. A 5-ml blood sample was taken at appropriate time intervals. The samples were immediately centrifuged, and the plasma was separated and kept frozen (−20°) until time for analysis.

RESULTS AND DISCUSSION

Gas–liquid chromatography

Watson *et al.*[6] used the liquid stationary phase OV-210 which is relatively non-retentive to achieve optimum separation with minimal tailing of SA, ASA and

internal standard. However, each "chromatographic run" took no less than 40 min. Similar results have also been observed in our laboratory. It is obvious that this assay method is very time consuming; therefore, the possibility of finding a better stationary phase which can separate the methyl esters in a shorter period of time was explored. A 3% OV-17 phase was found to serve the purpose. By increasing the oven temperature to 145°, each elution process was shortened to 8 min. As shown in Fig. 1a and b the peaks at 1.80, 4.44 and 6.40 min corresponded to the methyl esters of SA, internal standard and ASA respectively. No endogenous interference was observed when blank plasma samples were analysed.

Watson *et al.*[6] observed that the slow formation of an artifact had a retention time similar to the methylated internal standard when samples of methyl aspirin were left at room temperature. Similar findings were also obtained in our laboratory. In this study, the decomposition of the methyl esters was shown to be suppressed by keeping all the samples at 0°. This was substantiated by insignificant change of the slopes of the calibration curves and peak areas 72 h after methylation, when samples were kept cold.

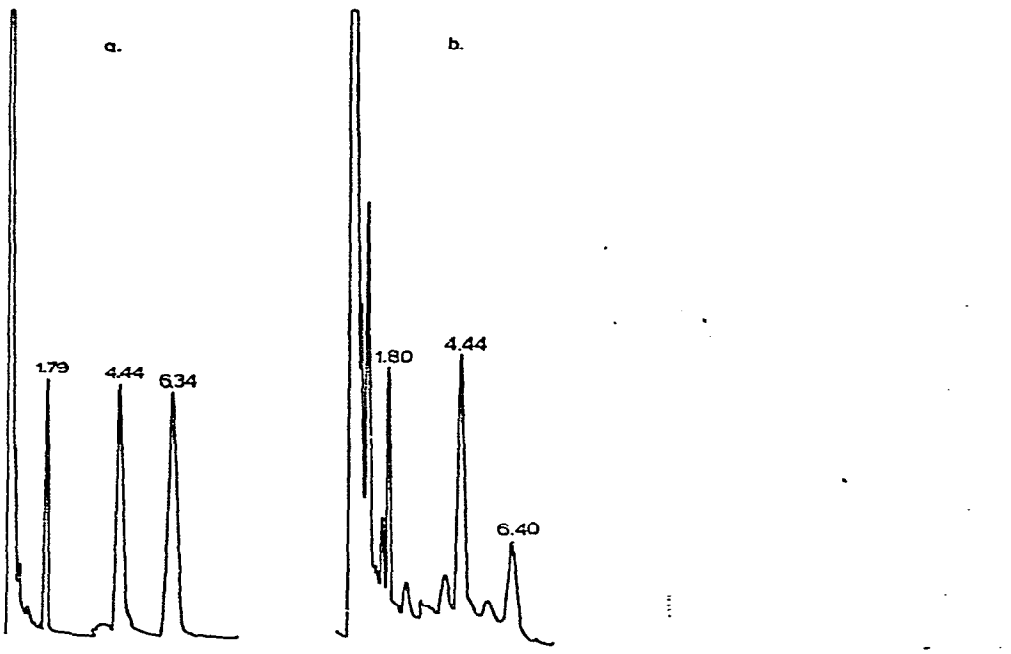

Fig. 1. Representative chromatograms of (a) methyl esters of SA, internal standard and ASA obtained from standard samples (retention times: 1.79, 4.44 and 6.34 min, respectively); (b) a plasma extract. Peaks at 1.80, 4.44 and 6.40 represent the methyl esters of SA, internal standard and ASA respectively.

Derivatization

Hexamethyldisilazane and trimethylchlorosilane[7], boron trifluoride methylating agent[8] and diazomethane[6] have been used to derivatize both ASA and SA. Among them, diazomethane was found to be the least time consuming because derivatization

takes place almost instantaneously. However, diazomethane is one of the most hazardous reagents used[10]. Its tendency to explode requires extra care in handling and investigators may tend to avoid this compound. In our laboratory, it was found that Levitt's microgeneration method[9] reduces the hazard of diazomethane, provided proper precautions are taken, because only a small amount was produced each time and the end product was consumed immediately. The set-up of the diazomethane generator was relatively easy and the addition of Diazald (25–250 mg) and the reaction mixtures only took a few minutes. The point that we are trying to establish is that in spite of the hazardous nature of diazomethane, it is still the reagent of choice owing to its convenience in this particular instance.

Calibration curve

As shown in Figs. 2 and 3 linearity is observed in the range of concentrations studied (5–50 μg/ml chloroform or plasma for SA and 0.5–30 μg/ml for ASA in

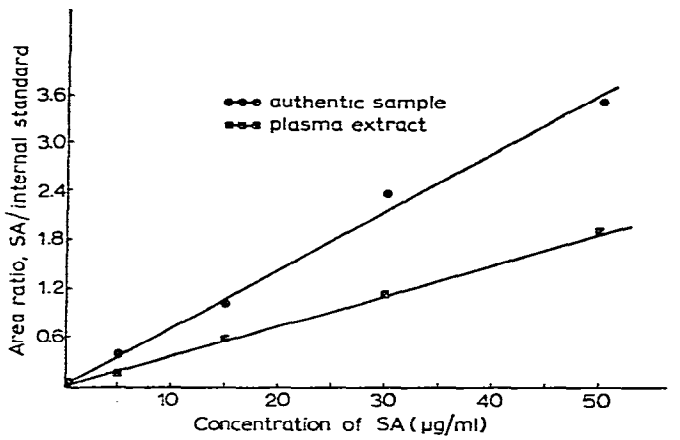

Fig. 2. Calibration curves of SA prepared from standard and spiked samples.

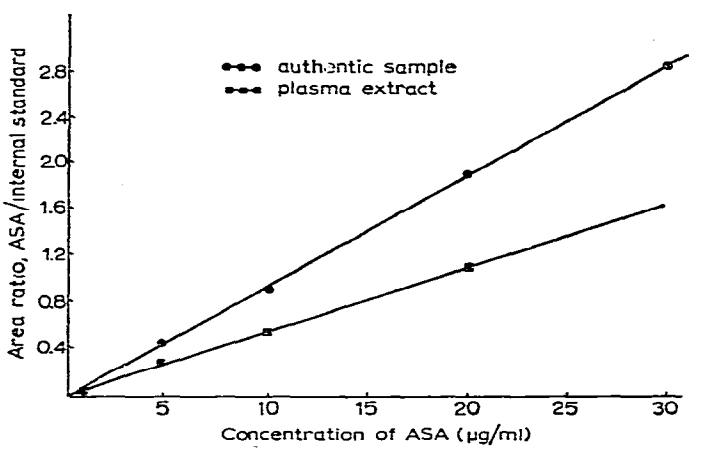

Fig. 3. Calibration curves of ASA prepared from standard and spiked plasma samples.

chloroform or plasma, with $r^2 = 0.99$ in all cases). The extraction efficiency of SA and ASA after conversion of the amount of SA or ASA in the aliquot (5 ml) to the original volume of organic layer is 71 and 79% respectively.

Estimation of SA and ASA in tablet dosage form and human plasma

Table I shows that the label claim for ASA per tablet was 300 mg. The estimated result indicated there was 93.50 ± 0.26% of the label claim when this GLC assay was used. About 2.3% of ASA in the tablet was hydrolysed to SA. This was not due to the on-column hydrolysis of ASA when this particular set of GLC conditions were employed, because a methylated sample of pure ASA did not produce any extra peaks. Similar results could also be reproduced using Watson's[6] method (Table I).

The applicability of this assay method in human plasma has been shown by studying the time course of effervescent aspirin tablets in a healthy human subject (Fig. 4). Peak plasma ASA concentration was achieved in 20 min followed by a rapid decline of the plasma drug level. SA, the major metabolite of ASA, kept on accumulating and reached a plateau at about 60 min. These observed plasma profiles for SA and ASA were almost identical to results reported by Rowland *et al.*[11].

TABLE I

A COMPARISON BETWEEN WATSON'S AND THE PRESENT MODIFIED METHOD IN QUANTITATIVE ESTIMATION OF ASA TABLETS

	Present method		*Watson's method*	
	ASA	*SA*	*ASA*	*SA*
Label claimed (mg/tablet)	300	0	300	0
Experimental (mg/tablet)*	280.53 ± 0.73	2.28 ± 0.004	288.75	3.5119 ± 0.80

* Each value is the average of two determinations.

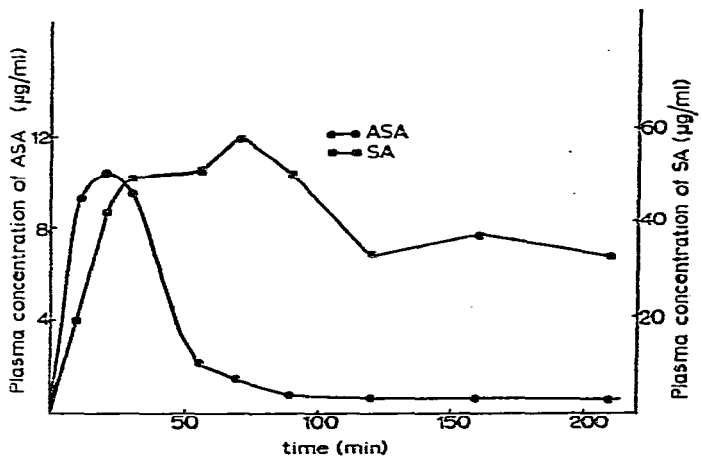

Fig. 4. Plasma concentration *versus* time plots of SA (■) and ASA (●) from a normal healthy volunteer.

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