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Note

Rapid and sensitive method for the determination of salicylic acid in serum by reversed-phase ion-pair high-performance liquid chromatography

STEPHEN T. INGALLS, JOHN F. SCHWERKOSKE, WALTER B. FORMAN and CHARLES L. HOPPEL*

*Departments of Pharmacology and Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106 (U.S.A.) and Medical Research Service, Veterans Administration Medical Center, Cleveland, OH 44106 (U.S.A.)**

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We have undertaken several studies which involve antithrombotic and non-steroidal anti-inflammatory agents. Most of these studies required patient abstinence from ingestion of pharmaceutical preparations containing salicylic acid. We required a method of salicylate determination suitable for application to the large number of serum specimens to be screened during the course of these projects. The short serum half-life of salicylate in man [1] complicated this requirement, since sensitivity far greater than that necessary for therapeutic salicylate level determination would be essential for detection of salicylate in serum more than 24 h after ingestion. The serum salicylate assay methods currently available include colorimetric methods [2—4], gas chromatographic [5, 6], thin-layer chromatographic [7], and high-performance liquid chromatographic (HPLC) procedures [8—18]. The colorimetric methods are limited by both a lack of specificity for salicylic acid [2—4] and marked inter-laboratory variation in results [19]. Gas chromatography of salicylic acid requires sample derivatization prior to separation [5, 6] which limits its convenience in handling large numbers of samples. High-performance thin-layer chromatography is less sensitive than modern HPLC determination methods and suffers from poor reproducibility [7]. Some authors describe HPLC assay methods which provide detection limits for salicylate in serum of 1 μg/ml, which would be adequate for screening applications [9, 12—15, 17, 18].

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However, none of these methods is adequately rapid for routine application to large numbers of samples. We also noted in preliminary trials with some of these separation procedures that salicylate often was unresolved from comparatively large quantities of endogenous interfering serum substances.

This paper presents a method for determination of serum salicylate involving extraction of salicylate and an internal standard from acidified serum, separation from interfering compounds by reversed-phase ion-pair HPLC, and absorbance detection at 301 nm. The detection limit corresponds with 45 ng injected into the chromatograph, and the total chromatographic separation time is 4.2 min. The reversed-phase ion-pair separation mechanism provides excellent chromatographic selectivity for salicylic acid and the internal standard with respect to both endogenous interference and 27 therapeutic drugs tested.

EXPERIMENTAL

Materials

Oxalic acid, tetrabutylammonium hydrogen sulfate, salicylic acid, and 3-methylsalicylic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sulfuric acid, sodium hydroxide, and ethyl acetate were purchased from Fisher Scientific (Cleveland, OH, U.S.A.). Acetonitrile (non-spectro grade) and methanol were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Equipment

The liquid chromatograph consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model M6000A pump, WISP 710B automatic sample injector, and RCM-100 radial compression module. A precolumn constructed from zero dead volume chromatographic unions (Crawford Fitting Co., Solon, OH, U.S.A.) and packed with the reversed-phase pellicular chromatographic medium Co:Pell-ODS (Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column against particulate sample contamination. A Perkin-Elmer (Norwalk, CT, U.S.A.) Model LC-75 variable-wavelength absorbance detector was used to monitor the absorbance of the eluent stream; the detector output was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. All peak height and area measurements and quantitative calculations were performed by a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354 chromatographic data system.

The analytical separation was carried out on a 10 cm × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm nominal particle diameter, Waters Assoc.). The chromatographic mobile phase was aqueous 0.1 *M* oxalic acid + 0.005 *M* tetrabutylammonium hydrogen sulfate (pH 4.00)—acetonitrile (75 : 25). The pH of the aqueous portion of the mobile phase was adjusted with solid sodium hydroxide prior to the addition of acetonitrile. The solvent pump was operated at a flow-rate of 5.0 ml/min.

Sample preparation

Standard solutions were prepared over a concentration range of 0.6—20 μg/ml by addition of measured quantities of salicylic acid stock solution to

chromatographically proven salicylate-free human serum. Serum aliquots of 500 μl were pipetted into 150 × 16 mm glass culture tubes. To these were added 50 μl of a 50 μg/ml solution in methanol—water (50:50) of the 3-methylsalicylic acid internal standard and 1 ml of 1 *M* sulfuric acid. The tube contents were vortexed briefly, 5 ml of ethyl acetate added, and the tubes vortexed continuously for 2 min. The tubes were centrifuged for 10 min at 1500 *g* to facilitate phase separation. The organic phase was transferred to clean 150 × 16 mm test tubes and evaporated to dryness at room temperature under a stream of dry, oil free compressed air. The residues were reconsituted in 200 μl of methanol—water (50 : 50) and 30-μl aliquots of this solution were injected into the chromatograph.

Reproducibility and recovery demonstration

Injection reproducibility was demonstrated by ten replicate injections of a solution containing both salicylic acid and the internal standard. Extraction efficiency and recovery experiments were performed by addition of 5-μg quantities of salicylic acid to the usual 500-μl serum aliquot, extraction, reconstitution in 200 μl of methanol—water (50 : 50) containing 5 μg 3-methylsalicylic acid, and injection of a 30-μl aliquot into the chromatograph. The salicylic acid: 3-methyl-salicylic acid peak height and area ratios obtained with experimental specimens were compared with the ratios obtained upon injection of a 30-μl aliquot of a methanol—water (50 : 50) solution containing 25 μg/ml each of salicylic acid and 3-methyl-salicylic acid; thus, this reference solution aliquot contained the absolute quantity of salicylic acid expected to be present upon complete extraction of salicylate from the experimental serum specimens. Apparent variations in extraction efficiency among the solvents investigated for experimental suitability were considered to be insignificant when smaller than the demonstrated injection reproducibility.

RESULTS AND DISCUSSION

We were unable to duplicate the recovery and reproducibility claimed by the authors of several published liquid chromatographic serum salicylate determination procedures. Experiments designed to investigate the dependence of extraction efficiency upon the identity and concentration of the mineral acid used for sample acidification were conducted. No significant differences in recovery were noted among experimental groups of six specimens acidified with 2 *M* sulfuric, 1 *M* sulfuric, 0.6 *M* hydrochloric, 1.2 *M* hydrochloric, 3.5 *M* phosphoric, 7% perchloric, and 14% perchloric acids and subsequently extracted with any one of the organic solvents used. The use of perchloric acid resulted in a flocculent precipitation of serum protein which was difficult to separate cleanly from the organic phase after sample extraction. Significant variation in the recovery of salicylic acid with the identity of the organic solvent used for extraction of serum aliquots acidified with 1 *M* sulfuric acid was observed. These results are presented in Table I. The recovery of salicylic acid from acidified serum containing 10 μg/ml of salicylate and extracted according to the outlined procedure was 95 ± 4%.

TABLE I

SALICYLIC ACID RECOVERY WITH SELECTED ORGANIC SOLVENTS

Recovery of 10 μg/ml salicylic acid from 500-μl serum aliquots acidified with 1 ml of 1 *M* sulfuric acid and extracted with 5 ml of solvent.

Solvent	Recovery (%) (n = 6)
Diethyl ether	17.9 ± 3.4
Toluene	41.1 ± 2.7
Butyl acetate	36.2 ± 7.8
Ethyl acetate	94.7 ± 3.6
Chloroform	72.4 ± 4.9
Isooctane—isopropanol (85 : 15)	37.8 ± 6.3

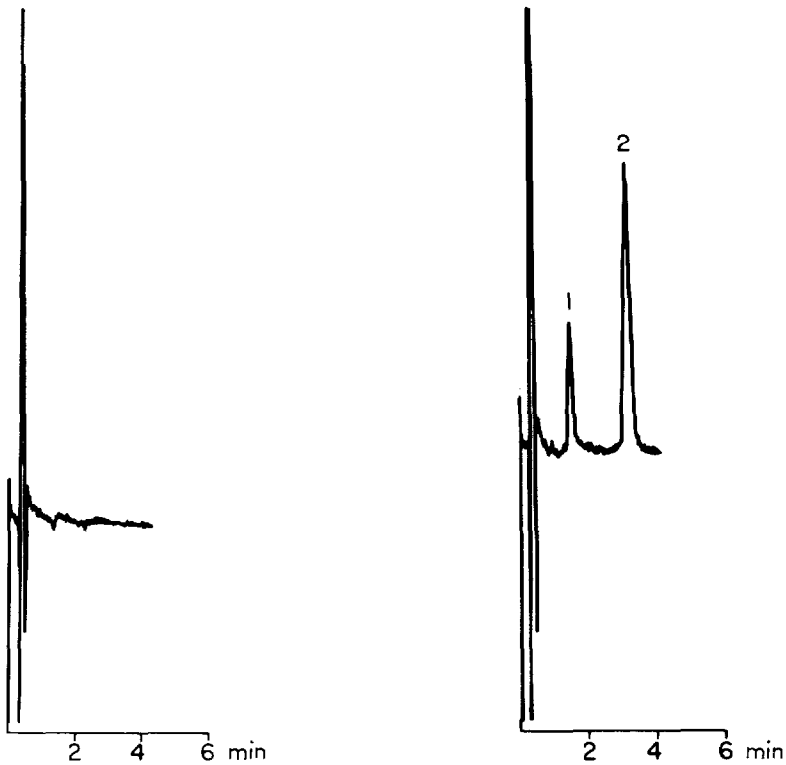

Fig. 1. Chromatogram obtained upon extraction of a salicylate-free serum specimen without added internal standard according to the outlined experimental procedure. The column was a 10 cm × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm nominal particle diameter). The chromatographic eluent was aqueous 0.1 *M* oxalic acid + 0.005 *M* tetrabutylammonium hydrogen sulfate (pH 4.00)—acetonitrile (75 : 25) and was pumped at a flow-rate of 5.0 ml/min. The absorbance detector was operated at 301 nm. The full scale of the ordinate is 0.005 absorbance units.

Fig. 2. Chromatogram obtained upon preparation of a serum aliquot containing 1.25 μg/ml salicylic acid and internal standard according to the described method. The chromatographic conditions were as described in Fig. 1. Salicylic acid (1) was eluted at 1.6 min; the internal standard 3-methyl-salicylic acid (2) was eluted at 3.2 min.

The chromatographic eluent system used was a slight modification of one developed by Waters Assoc. for quality assurance of an over-the-counter pharmaceutical preparation [20]. A chromatogram of a processed salicylate-free serum specimen is shown in Fig. 1. Fig. 2 is a chromatogram of a processed serum sample containing 1.25 μg/ml salicylic acid and added internal standard. The detection limit for salicylate (at a signal-to-noise ratio greater than 5 : 1) was found to correspond with 45 ng injected into the chromatograph. Especially noteworthy are the complete chromatographic separation time of 4.2 min, the extreme stability of the instrument signal baseline at maximum detector sensitivity, and the selectivity of the eluent and column combination for the compounds of interest relative to endogenous or medicinal interference (Table II). We infrequently noticed a chromatographic peak of an endogenous compound present in some serum specimens; this was eluted prior to salicylate and was completely resolved from the salicylate peak when both were present. The column has been used for assay development and routine determinations of more than 1600 serum specimens without undergoing noticeable deterioration in performance.

TABLE II

DRUGS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Duplicate 30-μl injections of each drug at a concentration of 1 mg/ml in methanol—water (50 : 50).

Non-steroidal anti-inflammatory agents	Anti-neoplastic agents	Miscellaneous
Phenylbutazone	5-Fluorouracil	Allopurinol
Naproxen	Doxorubicin	Theophylline
Fenoprofen	Vincristine	Procainamide
Ibuprofen	Vinblastine	Quinidine
Tolmetin	Cyclophosphamide	Furosemide
Zomepirac	Mutamycin	Phenytoin
Sulindac	Vindesine	Phenobarbital
Indomethacin	Cytosine arabinoside	Carbamzepine
Aspirin(O-acetylsalicylic acid)	Carmustine (BCNU)	Dipyridamole

Standard curves of salicylic acid : internal standard peak height ratios vs. sample salicylate concentration were linear (r^2 = 0.993) with a slightly positive y intercept of 0.05; this intercept value was less than 20% of the peak height ratio obtained for the standard solution containing the smallest salicylate concentration used for establishment of standard curves. The relative standard deviation for ten replicate injections of a single prepared serum specimen containing 10 μg/ml salicylate was 1.7%. The relative standard deviation in ten replicate determinations performed upon a serum specimen containing 10 μg/ml salicylate was 3.9%.

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