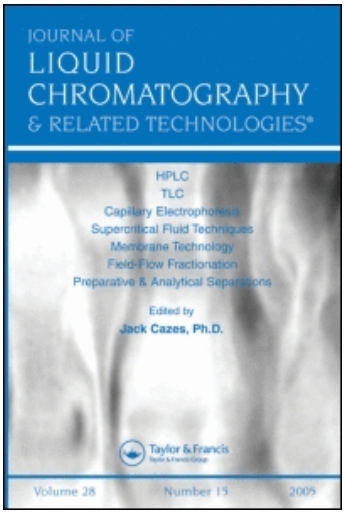

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ANALYSIS OF ACETYLSALICYLIC ACID AND ITS METABOLITES BY LIQUID CHROMATOGRAPHY

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ABSTRACT

The salicylates are the most commonly used analgesic, antipyretic and anti-inflammatory drugs. Measurement of serum levels of salicylate is important not only in the diagnosis and management of patients poisoned with salicylate, but also in therapeutic drug monitoring of arthritic patients on high dose salicylate therapy. While high performance liquid chromatography (HPLC) assays for these routine clinical measurements offer no distinct advantage over the colorimetric assays, they are indispensable in studying aspirin metabolism because of the ease with which HPLC assays can simultaneously measure aspirin and its metabolites. The superior sensitivity of HPLC assays compared to other techniques is needed for pharmacokinetic studies and for monitoring serum aspirin and salicylate levels following administration of low doses of aspirin for its anti-platelet aggregation effect. This paper will review the most recently published HPLC salicylate assays in terms of specimen collection and preparation, chromatographic conditions, and the different requirements of pharmacokinetic and clinical uses of salicylate measurements.

INTRODUCTION

Salicylate is one of the least expensive and most widely used drugs. The main therapeutic uses of aspirin to reduce pain, fever and inflammation have been known for many years. Hundreds of preparations of salicylates are in use, many of which are available to the consumer as over-the-counter home remedies[1]. The easy access of salicylate as a household item and the perception of it as a safe drug have been the cause of many drug overdose cases, particularly in the very young and the elderly.[2] Salicylate remains for many rheumatologists the drug of choice, or at least the initial drug for treating rheumatic diseases, despite the availability in recent years of newer non-steroidal anti-inflammatory drugs.[3] The efficacy of high dose salicylate for the managment of these patients is greatly aided by monitoring of serum salicylate levels.[2] This is because of the narrow therapeutic range and the large variation in dose-serum level relationships among patients. Therefore, the measurement of serum salicylate levels is an important clinical laboratory service in support of the management of patients intoxicated with salicylate and patients who are on high dose salicylate therapy.[2]

Although the therapeutic effectiveness of salicylate was appreciated for many years, the first understanding of the metabolism and mechanism of action was more recent. The molecular action of aspirin on prostaglandin biosynthesis was reported in 1971,[4] and only in the last 10 to 15 years have the complex pharmacokinetics of salicylate been described; much is not yet understood.[5] More recently, aspirin was shown to be inhibitory of platelet thromoxane

synthesis and therefore of platelet aggregation. This finding has led to intensive research into the use of aspirin in low doses to prevent formation of thrombi in coronary and cerebral arteries.[6] Thus research on the metabolism and pharmacokinetics of salicylate as well as the new therapeutic use of aspirin require assays of aspirin and its metabolites which are both sensitve and specific.

Techniques for Salicylate Measurement

Many techniques for salicylate measurement are available. They include colorimetric, fluorometric, enzymatic, gas liquid chromatographic and high performance liquid chromatographic assays.[2] The large group of colorimetric assays are based on the complexation of salicylic acid with ferric iron to give a purple color. The two popular colorimetric assays are Trinder's[7] and Natelson's;[8] practically all of the current colorimetic asays in use are these two methods or their modifications.[9] This reaction is not specific for salicylic acid, and some colorimetric assays have higher serum blanks than others. Furthermore, quantitation of aspirin requires differential measurement of salicylic acid before and after hydrolysis of aspirin to salicylic acid. The fluorometic[10] and enzymatic assays,[11] while proving to be more specific for salicylic acid, suffer from the disadvantage of not able to measure aspirin and its metabolites simultaneously. They also lack the sensitivity required for pharmacokinetic studies or for the monitoring of the anti-thrombotic use of low dose aspirin.

Gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) are the only techniques which are

quantitative, specific and sufficiently sensitive to measure low concentrations of aspirin and salicylic acid simultaneously. Optimized GLC assays, however, require derivatization of salicylate to overcome the low vapor pressure of salicylate and the tailing caused by polar functional groups.[12,13] This disadvantage is the reason for the current preference of HPLC over GLC for quantitative and specific measurement of the salicylates.

High Performance Liquid Chromatography

Of all the techniques that are currently in use to measure salicylate levels, high performance liquid chromatogrpahy (HPLC) is the most sensitive and specific.[14-33] A table summarizing 20 of the most recently published HPLC procedures is presented in (Table 1). The most sensitive of the HPLC methods claimed a detection limit of 0.01 mg/l, which is two orders of magnitude more sensitive than fluorometric or gas liquid chromatographic procedures.[14] The mobile phases are mostly methanol or acetonitrile. The pH values of these mobile phases are adjusted with either acetic acid or phosphate buffer to fall within the range of 2.5 - 3.0. One procedure using 35% methanol gave optimal resolution of aspirin and its metabolites to be at pH 3.9.[19] Aspirin and its metabolites have several absorption maxima, the one near 234 - 238 nm being most sensitive with the molar absorptivity of salicylic acid at 237 nm about 5 times that at 280 nm.[23] A majority of the plasma or serum assays have adopted this wavelength. The wavelength range of 300 - 330 is more suitable for urine specimens because of numerous endogenous acidic components present in urines that interfere with the analysis of

ASSAYS FOR SALICYLATES

REF.	ANALYTES[1]	SPECIMEN[2]	MOBILE PHASE[3]	NM[4]	TEMP	INTERNAL STANDARD	SENSITIVITY[5]
14.	SA, ASA	P	MEOH(27)1-BUOH(1)H_2O(72)H_3PO_4(0.013)	234	47	m-ANISIC ACID	0.5/0.01
15.	OTHERS	LINIMENT					
16.	SA, ASA	S	ACN(25)MEOTH(10)H_2O(65),pH 3	234			
17.	SA, ME-SA	B	MEOH(40)H_2O(66)HAC(1)	250		β-OH-ETHYLTHEOPHYLLINE	10
18.	SA, ASA, GA, SUA	P,U	MEOH(16)ACN(68)5nM PO_4(68), pH 2.5	237 330		MEPHENYTOIN	0.2/0.2
19.	SA, ASA, GA, SUA	S,P,U	MEOH(35)KH_2PO_4(63), pH 3.9	235	RT	TRIMETHOXYBENZALDEHYDE	1/1
20.	SA, ASA, GA, SUA	P,U	MEOH(35)H_2O(64)HAC(1)	238 305	RT	o-TOLUIC ACID (P), o ANISIC ACID(U)	0.5/0.5
21.	SA, ASA, GA, SUA	P,U,T	MEOH(40)H_2O(60), pH 3	238	45		0.25/0.5
22.	SA, ASA, SUA	P,U	ACN(25)H_2O(35)0.2M PO_4, pH 2.5(40)	234	RT	m-OH BENZOIC ACID	0.1/0.05
23.	SA, ASA, GA, SUA	P,U	ACN(30)0.03% H_3PO_4(70), pH 2.5	237		p-TOLUIC ACID	0.1/0.1
24.	SA, ASA	T	5% HAC, HEPTANE				
25.	SA, ASA, SSA	P,U	MEOH(60)0.1% HAC(40)	280		a-PHENYLCINNAMIC ACID	1
26.	SA, ASA	P	MEOH(55)0.072% H_3PO_4(45)	234	40	3,4 DIMETHYLBENZOIC ACID	0.5/0.5
27.	SA, ASA	P	MEOH(22)H_2O(73)HAC(5)	280	RT		5/2
28.	SA, SSA	P	MEOH 60% (99)HAC(1)	280		PHENYLBUTAZONE	
29.	SA, GA, SUA	P	MEOH(18)H_2O(77.9)HAC(4.1)	313	RT	o-ANISIC ACID	0.02
30.	SA, ASA, SUA	P	ACN(30)0.03% H_3PO_4(70), pH 2.5	237	50	PHTALIC ACID	0.5/0.5
31.	SA	S,P	MEOH(50)20mM HNO_3(50)	F	RT	o-ANISIC ACID	
32.	SA	S	$HCLO_4$	235			
33.	SA, ASA	T					

[1]SA, ASA, GA, SUA, SSA are salicylic acid, acetylsalicylic acid, gentistic acid, salicyluric acid and salicylsalicylic acid respectively. Others referes to SA, GA, SUA, 3-phenylpropylsalicylic acid, ethyl-5-methoxysalicylic acid and 5-methoxysalicylic acid.

[2]P, S, B, U, T are plasma, serum, blood, urine and tablet, respectively.

[3]MEOH, BUOH, ACN, HAC are methanol, butanol, acetonitrile, acetic acid, respectively, with percentage composition of each in parentheses.

[4]wavelength in nanometers (nm); second wavelength for urine assay F refers to fluorometry.

[5]sensitivity of assay for salicylic acid/acetylsalicylic acid in mg/l.

salicylate metabolites at 238 nm.[20] Many different compounds, most of which are derivatives of benzoic acid, have been selected as internal standards. Since the requirements of salicylate assays for pharmacokinetic studies and those for clinical use are different, they will be discussed separately.

HPLC Salicylate Assays for Pharmacokinetic Studies

Asprin or acetylsalicylic acid (ASA) is rapidly hydrolyzed to salicylic acid (SA) by esterase activities present at the intestinal wall, liver and other tissues.[34] The plasma half-life of aspirin is of the order of 15 - 20 minutes.[35] Thus, in order to follow plasma aspirin levels for 4 to 5 half-lives in pharmacokinetic studies, a sensitive assay which can determine very low concentrations of aspirin (< 1 mg/l) will be required. Salicylic acid is biotransformed by conjugation with glycine to form salicyluric acid (SUA), with glucuronic acid to form acyl and phenolic glucuronides (SAG and SPG). Salicylic acid is also hydroxylated to gentistic acid (GA), which itself is either conjugated with glycine to give gentisuric acid (GUA) or is glucuronidated.[5] Thus, HPLC assays for use in pharmacokinetic studies need to be able to measure not only ASA and SA, but also the metabolites SUA and GA in plasma and urine. Since aspirin is almost completely excreted by the kidneys (99%) as SA or its metabolites, in recovery studies, the amount of "total" salicylate can be measured in urine. The conjugated metabolites SUA, SPG, SAG and GUA can be hydrolyzed to salicylic acid and GA by heating acidified urine at 120°C for 1 to 3 hours.[22,23] Alternatively, the glucuronide metabolites

(SPG, SAG, GA) can be enzymatically hydrolyzed by glucuronidase.[18] Since SUA and GUA are glycine conjugates, they are not hydrolyzed by glucuronidase. Therefore, the HPLC assay used to measure "total" salicylate in enzymatically treated urine should be able to measure not only SA and GA, but also SUA and GUA.

Aspirin is rapidly hydrolyzed by esterases in blood ($t_{1/2}$ ~ 90 min) or plasma (40% hydrolyzed in 120 min) to salicylic acid.[27] Measurement of aspirin in studying the metabolism of aspirin is meaningful only if in vitro hydrolysis of aspirin to salicylic acid is inhibited. Therefore, blood specimens should be collected in chilled tubes containing an esterase inhibitor. Esterases in plasma are inhibited by sodium or potassium fluoride[36] and the cholinesterase inhibitor physostigmine,[27] although some workers found fluoride not to be very effective.[27] Blood cell esterases, however, are not inhibited by physostigmine, thus necessitating the prompt separation of blood cells from plasma to minimize destruction of aspirin, in vitro.[27] In addition to enzymic hydrolysis is acid-base hydrolysis of aspirin which account for 5% hydrolysis at room temperature and 10% loss of aspirin in frozen (-20°C) plasma over 24 days.[27] Thus, plasma specimens should be analyzed as soon as possible.

Preparation of serum or plasma specimens for chromatography can be either by protein precipitation or solvent extraction. Protein precipitation can be achieved by equal volumes of acetonitrile and plasma[20] or two volumes of acetonitrile[29] to ensure complete protein precipitation. A perchloric acid-methanol precipitating agent has also been used to yield cleaner baseline and to avoid the use of a

high speed centrifuge for the sedimentation of precipitated proteins.[19,23] The extraction of salicylate and its metabolites is greatly improved by the addition of methanol to perchloric acid.[23] HPLC assays which have the best sensitivity have incorporated a solvent extraction step followed by the evaporation of the organic solvent.[14,18,22,26,30] During evaporation, unpredictable loss of salicylate and aspirin due to sublimation has been reported.[18,25,30] This loss can be minimized by performing the evaporation step at low temperature.[26,30] One procedure circumvented the evaporation step by performing a back-extraction of the acidic organic phase with an alkaline aqueous solution. The alkaline aqueous phase was then injected into the chromatograph.[23]

HPLC Assays for Clinical Use

The often cited therapeutic range for anti-inflammatory action of salicylate is 150 - 300 mg/l, although mild toxic symptoms, such as tinnitus, have been associated with plasma salicylate levels greater than 200 mg/l. Severe intoxication of aspirin is usually found with plasma levels greater than 500 mg/l.[2] Serum salicylate levels, in conjunction with Done's nomogram, are used extensively to predict the severity of intoxication of the ingested formulation of regular tablets.[37,38] Thus, non-chromatographic salicylate assays (e.g., Trinder's assay) are adequate for routine therapeutic drug monitoring or for the diagnosis and management of patients intoxicated with aspirin. Most of the published HPLC procedures were developed for use in pharmacokinetic studies. Thus, the sensitivities of these assays

are far beyond those needed for current clinical uses of serum salicylate levels. Furthermore, not all of the HPLC procedures have been investigated for interference by other drugs[14,17,21,23,27,29,31,32] since these assays were developed for use in pharmacokinetic studies which are conducted under standard and supervised conditions, and study subjects are not on other medications.[18-20,22,25,26,28,30] Thus the usefulness of these HPLC assays in the clinical settings is uncertain, and acceptance of these assays by clinical laboratories will require first testing them for possible drug interference. Assays which were developed for clinical purposes did not attempt to measure the metabolites of salicylate[16,17,26,27,31] or did not measure aspirin.[31]

HPLC procedures should be tested for their specificity in not measuring structurally related compounds and ketone bodies.[39] These substances in plasma can reach high levels in clinical conditions which present with metabolic derangements.[40] In this respect, it has been recommended that HPLC assays rather than colorimetric or fluorometric should be used to measure salicylate levels in patients with Reye's syndrome.[40] In patients who are severely overdosed with aspirin, the accumulation of aspirin and salicylate metabolites in plasma can be sufficiently high to cause significantly higher apparent salicylate concentration when it is measured by a non-specific colorimetric assay.[41]

A potential area for therapeutic drug monitoring of salicylate is in the use of aspirin as an anti-thrombotic drug.[6] The optimal aspirin dosage for anti-thrombotic therapy is yet to be agreed upon. There is

little doubt, however, that the dosage (e.g., <100 mg) will be considerably lower than those administered for antipyretic and analgesic purposes. Thus, following administration of antithrombotic doses of aspirin, aspirin and salicylate levels will be very low-less than 10 and 1 mg/l, respectively.[14] Quantitation of these low levels of aspirin and salicylate will demand analytical sensitivity that is met only by HPLC.

Data from animal studies have suggested that free salicylate rather than total salicylate correlates better with pharmacological activities of salicylate.[42] Salicylate is one of the few drugs which at therapeutic concentration saturate the binding sites of its binding protein (albumin).[43] Hence free salicylate concentration will change with total drug concentration and the extent of protein binding of the drug. In a study of free salicylate level of thirty patient sera submitted to the clinical chemistry laboratory for quantitation of total salicylate, total salicylate levels ranged from 62 to 731 mg/L and the corresponding free salicylate fraction ranged from 0.065 to 0.588 (Kwong and Baum, unpublished results). Therefore, with such a large variation in free salicylate levels among patients, a therapeutic drug monitoring program based on free salicylate rather than total salicylate is in principle superior. Equilibrium dialysis and ultrafiltration are the two techniques most applicable to the routine measurement of free drug.[44] Since salicylate at the low therapeutic concentration is > 90% bound to albumin,[42] the free salicylate concentration in either an equilibrium dialysate or an ultrafiltrate will be low (10 -20 mg/l). Such free salicylate concentrations are below

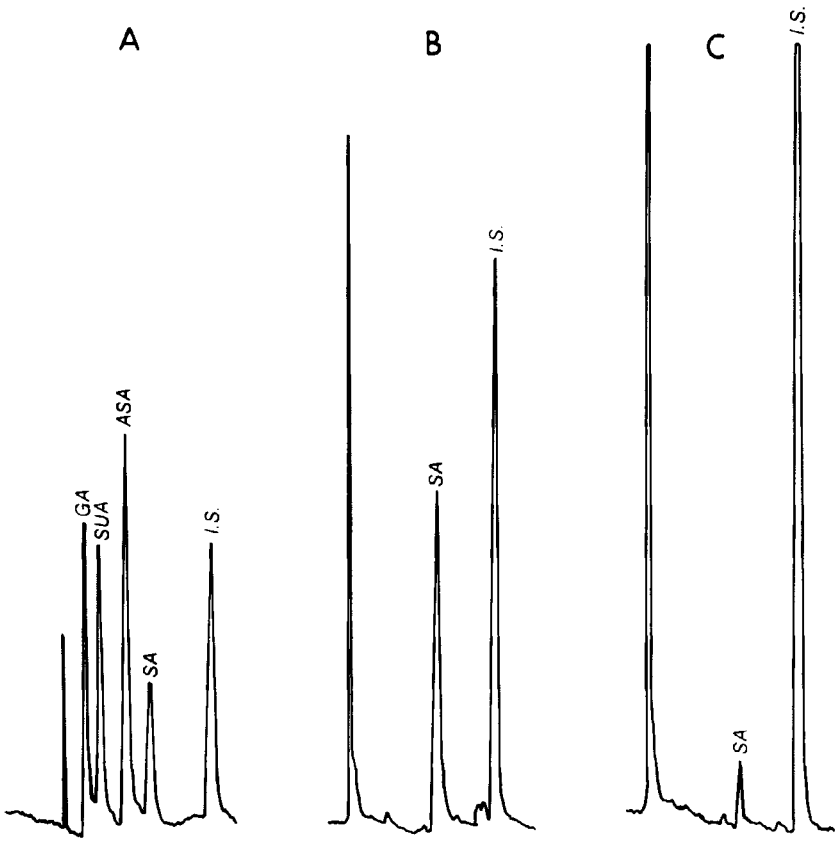

FIGURE 1

A. Chromatogram of serum spiked with 10 mg/L each of gentistic acid (GA), salicyluric acid (SUA) acetylsalicylic acid (ASA), salicylic acid (SA) and internal standard, 3.4 dimethylbenzoic acid (IS). The retention times are 2.0, 2.6, 3.7, 4.7 and 7.3 min. respectively.

B. Chromatogram of patient serum containing 44 mg/L of salicylic acid.

C. Chromatogram of an ultrafiltrate of the same patient serum containing 4.6 mg/L of salicylic acid.

Different integrator attenuation settings were used in Figure 1B and C.

Chromatography was carried out with a varian MCH 4 x 15 cm column at 45°C, 18 min/ml using a mobile phase of 24% acetonitrile in 60 mM phosphoric acid pH 2.3, and monitored at 237 nm.

the sensitivity limits of colorimetric assays. The quantitation of free salicylate levels will require the use of a sensitive HPLC assay. A HPLC method has been developed to measure low levels of salicylate in serum and ultrafiltrate (Kwong, unpublished results). This procedure uses a simple sample preparation step by acetonitrile[20] followed by high speed centrifugation (5 min) to pellet the precipitates. Chromatography of the supernatants consistently yield clean and stable baseline with the detection limit of salicylic acid at 0.5 mg/L. With suitable modification, this method can be used to measure aspirin and its metabolites. Figure 1A shows a chromatogram of a serum spiked with GA, SUA, ASA and SA. Chromatograms showing an ultrafiltrate containing 4.6 mg/l of salicylate obtained from ultrafiltration of a patient serum containing 44 mg/l of salicylate are reproduced in Figure 1B and 1C, respectively. This assay has been used in the author's laboratory to study protein binding of salicylate and the clinical significance of free salicylate levels in monitoring elderly arthritic patients.

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