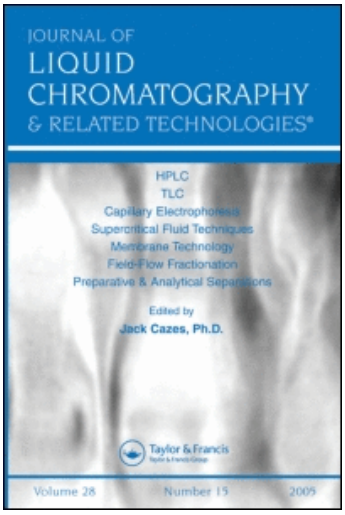

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Sensitive Assay for Aspirin and its Metabolites Using Reversed-Phase Ion-Pair High-Performance Liquid Chromatography

J. Shen[a]; S. Wanwimolruk[a]; M. S. Roberts[a]; C. R. Clark[b]
[a] Department of Pharmacy, University of Otago Medical School, Dunedin, New Zealand [b] Division of Medicinal Chemistry Department of Pharmaceutical Sciences, School of Pharmacy Auburn University, Auburn, Alabama

To cite this Article Shen, J. , Wanwimolruk, S. , Roberts, M. S. and Clark, C. R.(1990) 'A Sensitive Assay for Aspirin and its Metabolites Using Reversed-Phase Ion-Pair High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 4, 751 — 761
To link to this Article: DOI: 10.1080/01483919008051818
URL: http://dx.doi.org/10.1080/01483919008051818

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(4), 751-761 (1990)

A SENSITIVE ASSAY FOR ASPIRIN AND ITS METABOLITES USING REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. SHEN[1], S. WANWIMOLRUK[1], C. R. CLARK[2], AND M. S. ROBERTS[1]

[1]*Department of Pharmacy*
University of Otago Medical School
Dunedin, New Zealand

[2]*Division of Medicinal Chemistry*
Department of Pharmaceutical Sciences
School of Pharmacy
Auburn University
Auburn, Alabama 36849

ABSTRACT

An ion-pair high performance liquid chromatographic (HPLC) method was developed for quantitating aspirin (ASA) and its major metabolites (salicylic acid (SA), gentisic acid (GA) and salicyluric acid (SU)) in human plasma. The average extraction recovery for ASA and its metabolites was greater than 90%. The limit of sensitivity of this assay was 0.5, 0.05, 0.1 and 0.05 μg/ml for GA, SU, SA and ASA, respectively. The method gave high reproducibility with coefficients of variation less than 10%. This method has been used for monitoring SA and ASA levels in preeclamptic patients after administration of low dose aspirin.

INTRODUCTION

There has been an increasing interest in recent years in the use of low dose of aspirin as an anti-thrombotic drug. The optimal aspirin

dosage for anti-thrombotic therapy is yet to be agreed upon but appears to be approximately 100 mg daily (1). Thus, following administration of an anti-thrombotic dose of aspirin, aspirin and salicylate concentrations are very low even though platelet aggregation *ex vivo* (2-4) and cyclooxygenase (4-6) are inhibited. A more sensitive quantitative assay for these low concentrations of aspirin and salicylate is essential.

Various methods have been reported for quantitating aspirin and its metabolites in different biological media. These include the standard colorimetric (7) and fluorometric (8-9) methods and the modern analysis by gas-liquid chromatography (10) and high-performance liquid chromatography (HPLC) (11-23). Of these analytical methods HPLC presently is the most sensitive and specific. Most of the published HPLC procedures were developed for use in pharmacokinetic studies. These assays either do not provide adequate sensitivity for current clinical monitoring of anti-thrombotic use of low dose aspirin or are cumbersome to use. At present, there appear to be no reports on the potential of ion-pair HPLC as an analytical method for the quantification of aspirin and its metabolites in biological fluids. The present report describes a specific and sensitive method for the simultaneous determination of aspirin and its major metabolites using ion-pair reversed phase HPLC.

MATERIALS AND METHODS

Reagents

Aspirin (acetylsalicylic acid, ASA) was obtained from Halewood Chemicals Ltd. (Middlesex, England). Salicylic acid (SA), salicyluric acid (SU), gentisic acid (GA) and m-hydroxybenzoic acid (m-HBA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Triethylamine (TEA), phosphoric acid, methanol and ether were purchased from BDH Chemicals Ltd (Poole, England). Hexane was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were analytical grade.

Chromatographic Conditions

Reversed phase HPLC was performed using a Shimadzu Model LC-6A solvent pump, a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-µl loop and a µBondapak phenyl column (300 X 4.6 mm I.D., 10 µm average particle size, Waters Associates). The absorbance of the eluent was determined using a Shimadzu Model SPD-6AV UV-VIS Spectrophotometric detector. The absorbance was recorded on a Model TOZN1-H electronic recorder (Tohshin Electron Co., Ltd, Japan).

Conditions for optimal separation of the compounds of interest were studied using a mobile phase consisting of methanol, water and TEA (280/717.5/2.5, v/v/v, pH 3.5±0.05) in which the final pH of the mobile phase was systematically adjusted with concentrated phosphoric acid. After adjustment, the mobile phase was degased and filtered by membrane filters (0.45 µm, Millipore Corp., Bedford, MA, U.S.A.). The flow rate was 1 ml/min and the effluent was monitored at 229 nm. Standard curves were constructed based on peak height ratios obtained by internal standardization.

Assay Procedure for the Measurement of Aspirin and Its Metabolites In Human Plasma

A 0.5 ml plasma sample was pipetted into a test tube containing 0.5 ml of aqueous 1.0 M oxalic acid solution, 0.5 ml of 25 mM phosphate buffer (H_3PO_4/KH_2PO_4, pH 2.5), containing internal standard (m-HBA, 50 µg/ml), and other standards where appropriate for standard curve calibrations. The mixture was vortexed for 2 min with 5 ml of an equivolume solution of ether/hexane, then centrifuge at 3000 rpm for 3 min. The organic phase was transferred to another centrifuge tube containing 200 µl of 0.5 M phosphate buffer (KH_2PO_4/Na_2HPO_4, pH 7.0). The mixture was then vortexed for 2 min and centrifuge for 3 min. A 100 µl portion of the aqueous phase was transferred and mixed with 50 µl of 25% H_3PO_4 and a 20 µl sample of this mixture was injected onto the HPLC column.

RESULTS AND DISCUSSION

In this study a reversed phase HPLC method has been developed for the determination of plasma levels of GA, SU, SA and ASA in patients on low dose aspirin therapy. The ion-pairing technique shows improved resolution and sensitivity over previous methods (17).

Triethylamine (TEA) was chosen as the ion-pairing reagent to aid overcoming the analytical difficulties encountered from endogenous interfering compounds in plasma at very low concentration of aspirin and its metabolites. TEA has been used as a mobile phase modifier for the reversed phase HPLC separation of acidic drugs (24). Numerous reports have shown that the retention of a solute can be altered by incorporating TEA in the eluent to serve as a competing agent for masking accessible surface silanol groups(24). The effects of TEA on capacity factor, k', for the solute of interest, were investigated by varying the TEA concentration in the mobile phase. Figure 1 shows the results of that study. In general, excellent resolution of salicylates was obtained with 0.25% TEA and the interference by endogenous substances in plasma was largely decreased.

Plots of k' values against the concentration of methanol in the mobile phase (Figure 2) was used to determine the solvent composition for optimal resolution for the compouds of interest. The data show that as the concentration of methanol increases the retention time decreases. The mobile phase containing 28 % methanol gave the best resolution of GA, SU, SA, ASA and internal standard. At 28 % methanol the resolution between SU an SA is maximized and as shown in Figure 2 the resolution between these two compounds decreases at both higher and lower concentrations of methanol.

In our procedure, optimal resolution of the compounds of interest was achieved by adjusting the mobile phase pH to 3.5. This pH level is higher than the typical values of pH 2.5 to 3.0 reported (11-23) in previous studies. Although pH 3.5 should be high enough to allow considerable dissociation for many of the carboxylic acids, in this study no detrimental effects on chromatographic peak shape were observed.

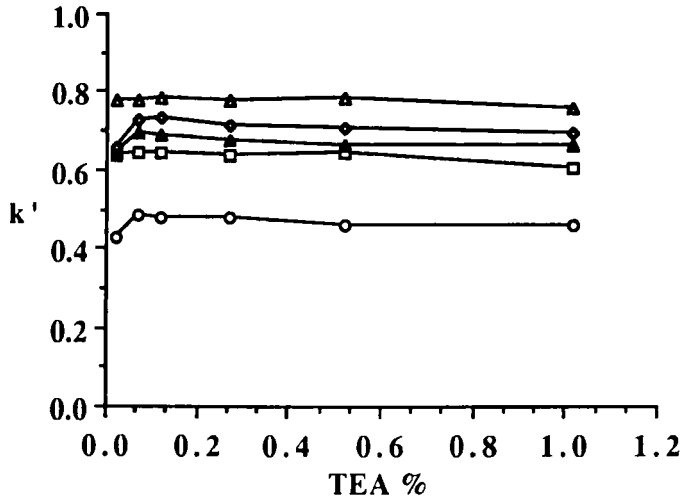

FIGURE 1 Effect of TEA concentration on the capacity factor (k') of GA (○), SU (▲), ASA (△), SA (◇) and m-HBA (□)

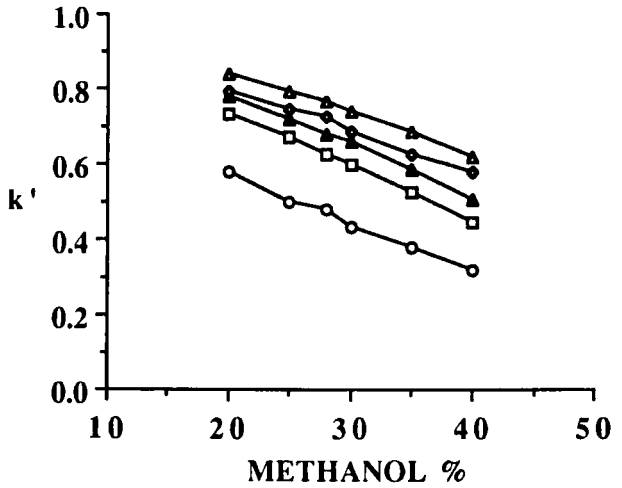

FIGURE 2 Effect of methanol concentration on the capacity factor (k') of GA (○), SU (▲), ASA (△), SA (◇) and m-HBA (□)

Aspirin and its metabolites have several ultraviolet absorption maxima, however the one in the 234-238 nm range has the highest molar extinction coefficient and is about 5 times that of the molar extinction coefficient at 280 nm (18). The wavelength of detection was chosen at 229 nm in this study which allows maximum sensitivity for aspirin and salicylic acid and gives an optimal absorbance for all other compounds of interest.

Unpredictable loss of aspirin and salicylate during evaporation can occur due to sublimation. Such loss was avoided by improving the back-extraction of the organic phase with an alkaline aqueous solution. The alkaline aqueous phase was acidified and then injected onto the chromatographic system for analysis. The best mobile phase for this method is an isocratic mixture of methanol, water and TEA (280/717.5/2.5, v/v/v, pH 3.5). Using this mobile phase running at a flow rate of 1 ml/min, the retention times of GA, SU, SA and ASA were 5.5, 8.6, 10.1 and 11.6 min, respectively. The extraction system utilized is both highly efficient and convenient. The extration of all salicylates from human plasma at different concentrations (1, 10 and 100 μg/ml, respectively) gave consistent recoveries (92-100%).

m-Hydroxybenzoic acid (m-HBA) was chosen as the internal standard in the present assay as it was completely resolved from the compounds of interest and blank plasma showed no peaks that interfered with m-HBA. Several compouds were examined in this system as internal standards and Table 1 shows the compounds tested and their capacity factors.

Interference by other drugs was determined by analysing plasma samples spiked with various drugs. None of the drugs listed in Table 2 interfered with the assay, furthermore the back-extraction avoided any interference by some normally used basic drugs.

The calibration curves for plasma ASA and SU were linear in the range 0.05 to 200 μg/ml while SA was linear from 0.1 to 200 μg/ml and GA from 0.5 to 200 μg/ml. The limit of detection was defined as signal-noise ratio (3:1). In Figure 3, typical chromatograms are shown, one from a healthy volunteer and another one from a preeclamptic woman after oral administration of 100 mg of aspirin (slow-release formulation, Cartia, SK&F).

Table 1
Relative Retention Time (k') of Compounds as Internal Standards and Salicylates

k'	Compound
0.89	dl-Mandelic acid
0.96	Gentisic acid
1.07	p-Aminosalicylic acid
1.07	p-Aminobenzoic acid
1.11	2,4-Dihydroxybenzoic acid
1.25	p-Hydroxyphenylacetic acid
1.29	Phthalic acid
1.32	p-Hydroxybenzoic acid
1.87	m-Hydroxybenzoic acid
2.14	m-Hydroxybenzaldehyde
2.18	Phenoxy-acetic acid
2.43	Salicyluric acid
2.86	Salicylic acid
3.39	Phenylacetic acid
3.39	p-Nitrobenzoic acid
3.61	Acetylsalicylic acid
6.18	p-Anisic acid
8.14	p-Toluic acid
8.25	m-Toluic acid
>10	3-Hydroxy-2-naphthoic acid

* k' is defined as (tr/to)-1, where to was measured as the first distortion of the base line following the injection of water.

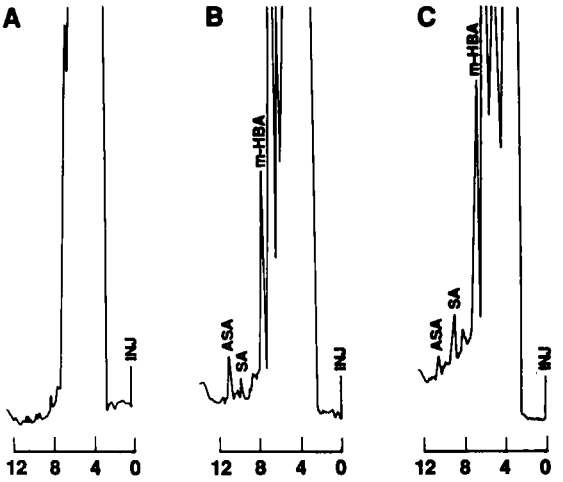

FIGURE 3 Chromatograms of (A) blank human plasma, (B) human plasma taken 1 hour after oral administration of 100 mg of aspirin (slow-release formulation, Cartia, SK&F). The concentrations found in this sample were 226 ng/ml for ASA and 70.3 ng/ml for SA and (C) a preeclamptic patient's plasma taken, at delivery, appropreciately 8 hours after oral administration of 100 mg of aspirin (Cartia, SK&F) daily for 6 months. The concentrations found in this sample were 50 ng/ml for ASA and 102 ng/ml for SA. The full scale of the ordinate is 0.002 absorbance units.

Table 2

Drugs Tested for Interference in Human Plasma

Acetaminophen	Ibuprofen	Phenytoin
Butobarbitone	Indomethacin	Prazosin
Cafein	Labetalol	Propranolol
Cocaine	Morphine	Quinine
Codeine	Naproxen	Theophylline

No interference for all drugs tested with aspirin assay

The intra- and inter-assay reproducibility of the method was investigated by analysing six plasma replicates of each compound at different concentrations (1, 10 and 100 μg/ml, respectively) over one month. The coefficients of variation for the peak height ratios of GA, SU, SA and ASA were less than 10% (2-7.9%). It was shown that the method is precise and reliable, demonstrating a good recovery with small variation.

In summary, the method reported here uses an ion-pairing reversed-phase HPLC technique to achieve high sensitivity and overcome the problem of interference by endogenous substances in plasma, and of normally used drugs in the presence of very low concentrations of aspirin and salicylic acid. The smaller sample size needed for our technique (0.5 ml plasma) permits use of small blood samples compared to other reported methods. This method can be used to monitor the anti-thrombotic use of low dose aspirin and in our laboratory it is being used to study the clinical significance of salicylate levels in preeclamptic patients.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. P. D. Wilson of Department of Obstetrics and Gynecology for collecting patient's blood samples.

REFERENCES

(1) Weksler B.B., Pett S.B, Alonso D., Richter R.C., Stelzer P., Subramanian U., Tack-Goldman K. and Gay W.A. Jr. N. Engl. J. Med., 308, 800, 1983

(2) Siebert D.J., Bochner F, Imhoff D.M., Watts S., Lloyd J.V., Field J. and Gabb B.W. Clin. Pharmacol. Ther., 308, 367, 1983

(3) Ross-Lee L.M., Elms M.J., Cham B.E., Bochner F., Bunce I.H. and Eadie M.J. Eur. J. Clin. Pharmacol., 23, 545, 1982

(4) Roberts M.S., McLeod L.J., Cossum P.A. and Vial J.H. Eur. J. Clin.Pharmacol., 27, 67, 1984

(5) Ali M., McDonald J.W.D., Thiessen J.J. and Coates P.E. Stroke, 11, 9, 1980

(6) Pedersen A.K. and FitzGerald G.A. N. Engl. J. Med., 311, 1206, 1984

(7) Muni I.A., Leeling J.L., Helms R.J., Johnson N., Bare J.J. and Phillips B.M. J. Pharm. Sci., 67, 289, 1978

(8) Graham G. and Rowland M. J. Pharm. Sci., 61, 1219, 1972

(9) Siebert D.M. and Bochner F. J. Chromatogr., 420, 425, 1987

(10) Tischio J.P. J. Pharma. Sci., 65, 1530, 1976

(11) Dadgar D., Climax J., Lambe R. and Darragh A. J. Chromatogr., 342, 315, 1985

(12) Tebbett I.R. and Omile C.I. J. Chromatogr., 329, 196, 1985

(13) Mays D.C., Sharp D.E., Beach C.A., Kershaw R.A., Bianchine J.R. and Gerber N. J. Chromatogr., 311, 301, 1984

(14) O'Kruk R.J., Adams M.A. and Philip R.B. J. Chromatogr., 310, 343, 1984

(15) Barkar S.K. and Niazi S. J. Pharm. Sci., 72, 1020, 1983

(16) Reidl U. J. Chromatogr., 272, 325, 1983

(17) Buskin J.N., Upton R.A. and Williams R.L. Clin. Chem., 28, 1200,1982

(18) Rumble R.H., Roberts M.S. and Wanwimolruk S. J. Chromatogr., 225, 252, 1981

(19) Harrison L.I., Funk M.L. and Ober R.E. J. Pharm. Sci., 69, 1268, 1980

(20) Lo L.Y. and Bye A. J. Chromatogr., 181, 473, 1980

(21) Cham B.E., Johns D., Bochner F., Imhoff D.M. and Rowland M. Clin. Chem., 25, 1420, 1979

(22) Peng G.W., Gadalla M.A.F., Smith V., Peng A and Chiou W.L. J.Pharm. Sci., 67, 710,1978

(23) Gaspari F. and Locatelli M. Ther. Drug Monitor., 9, 243, 1978

(24) Roos R.W. and Lau-Cam C.A. J. Chromatogr., 370, 403, 1986