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Note

Determination of plasma aspirin and salicylic acid concentrations after low aspirin doses by high-performance liquid chromatography with post-column hydrolysis and fluorescence detection

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There has been an increasing interest in recent years in the use of low doses of aspirin for anti-platelet therapy. This, and reports of inhibition of platelet aggregation ex vivo [1–3] and cyclooxygenase [3–5] even though plasma aspirin concentrations were low or unmeasurable, underline the need for more sensitive aspirin assays than are presently available.

Of the many published assays for aspirin in biological fluids very few have reported detection limits below 100 ng/ml [6–9]; a gas chromatographic–mass spectrometric assay [6] and a high-performance liquid chromatographic (HPLC) assay with UV detection [7], both with detection limits of 10 ng/ml, being the most sensitive of these. Rumble et al. [10], in an attempt to improve the sensitivity of their assay (detection limit 100 ng/ml), found that the fluorescence of aspirin was insufficient to provide better detection sensitivity than monitoring the UV absorbance at 237 nm. Although aspirin itself exhibits only weak fluorescence, it is rapidly hydrolysed in alkaline solution to salicylic acid which is strongly fluorescent, particularly at high pH. Therefore it should be possible, by incorporating a post-column hydrolysis step before fluorescence detection, to increase the sensitivity of HPLC assays for aspirin.

EXPERIMENTAL

Materials

Aspirin was obtained from BDH (Poole, U.K.) and salicylic acid and physostigmine hemisulphate from Sigma (St. Louis, MO, U.S.A.). All other reagents

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and solvents were of analytical grade. Methanol was redistilled before use. Phosphate buffer (10 m*M*; pH 7.4) was prepared using 10 m*M* potassium dihydrogenphosphate and disodium hydrogenphosphate solutions.

Sample preparation

Blood was collected into chilled 10-ml heparinised tubes containing 100 μl of a 40 m*M* physostigmine hemisulphate solution, the plasma separated immediately and stored frozen until assay within two weeks [11].

Plasma (1 ml) or plasma (0.5 ml) plus 10 m*M* phosphate buffer pH 7.4 (0.5 ml) was added to a screw-capped glass extraction tube followed by diethyl ether (3 ml) and 21.25% phosphoric acid (50 μl) (85% orthophosphoric acid–water, 1:3). The sample was mixed on a rotary mixer for 10 min and then centrifuged at 1500 *g* for 5 min. The ether phase was transferred to a capped centrifuge tube containing 10 m*M* phosphate buffer pH 7.4 (150 μl) and vortex-mixed for 1 min. After centrifugation and aspiration of the ether phase the aqueous layer was bubbled briefly with nitrogen to remove residual ether and kept on ice until 50 μl were injected into the chromatograph.

Standards were prepared by spiking blank plasma collected as above with concentrated solutions of the standard in phosphate buffer. Aspirin standard solutions were freshly prepared on the day of use.

Chromatography

The samples were chromatographed on a 5-μm Spherisorb ODS 2 (SGE Scientific, Melbourne, Australia) column★, 5 cm × 4.6 mm, which was packed using a slurry packing apparatus and then endcapped with HPLC column endcapping agent (Alltech Assoc., Deerfield, IL, U.S.A.). The solvent, 40% methanol and 0.085% phosphoric acid in water, was pumped through the column at a flow-rate of 1 ml/min by an M6000A pump (Millipore–Waters, Milford, MA, U.S.A.). Sodium hydroxide (0.5 *M*) at 0.15 ml/min (K25 pump, ETP Kortec, Sydney, Australia) was merged via a T piece (Alltech) with the eluent from the column which then flowed through a reaction coil constructed from 16 m × 0.25 mm I.D. stainless-steel tubing (HLS Scientific, Adelaide, Australia) with a coil diameter of 6 cm. The reaction coil was immersed in an oil bath thermostatted at 60°C. The separated and hydrolysed aspirin was then measured as salicylic acid by a fluorescence detector (Schoeffel FS970, Kratos, Westwood, NJ, U.S.A.) with an excitation wavelength of 310 nm and a 389-nm emission cut-off filter. A Curken-single-channel 10-mV recorder was used to record the signal. Sample aliquots (50 μl) were injected via a U6K injector (Millipore–Waters).

The assay reproducibility was assessed by spiking blank plasma with aspirin to concentrations of 5, 50 and 500 ng/ml or with salicylic acid to 50, 500 and 2000 ng/ml. Five 1-ml and five 0.5-ml aliquots at each concentration were extracted and assayed as described above, and the coefficient of variation at each concen-

★The initial plate count, *N*, of the column was 4875 calculated by the 5σ method for acenaphthene [10 μl of solution eluted with acetonitrile–water (60:40) at 2 ml/min]. The peak asymmetry was 1.25.

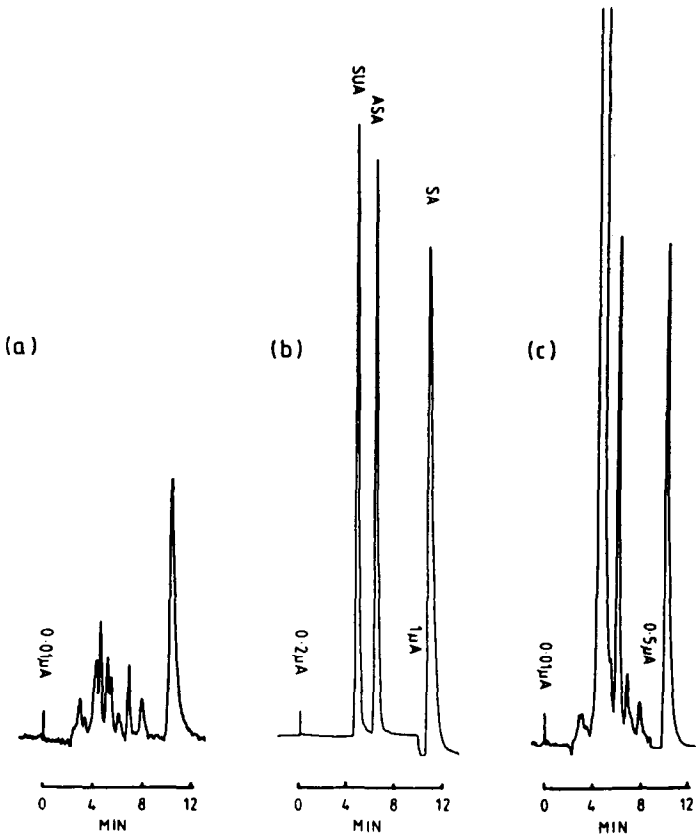

Fig. 1. Chromatograms of plasma extracts of samples taken (a) before, (b) 30 min after and (c) 2 h after 50 mg aspirin taken as an aspirin solution (Aspro Clear, Nicholas) by a volunteer. Aspirin and salicylic acid concentrations are 576 ng/ml and 3.6 μg/ml (b) and 18.5 ng/ml and 1.8 μg/ml (c), respectively. The sensitivity setting on the fluorimeter was 4.26. Peaks: SUA = salicyluric acid; ASA = aspirin; SA = salicylic acid.

tration was determined. Plasma from a number of patients on multiple-drug therapy was also assayed to check for interference with the measurement of aspirin by other drugs or their metabolites.

A pilot pharmacokinetic study was conducted to confirm the utility of the assay. A young male volunteer (aged 21 years, weight 54 kg) took 50 mg enteric coated aspirin granules (Astrix, F.H Faulding, Adelaide, Australia) each morning for one week. Immediately before and at intervals for 7 h after the final dose, blood samples were taken from an indwelling catheter (Jelco, Critikon, Tampa, FL, U.S.A.) for measurement of plasma aspirin and salicylic acid concentrations.

RESULTS AND DISCUSSION

The use of a post-column hydrolysis step before fluorimetric detection has enabled us to measure as little as 2 ng/ml aspirin in plasma. Fig. 1 shows chromato-

TABLE I

REPRODUCIBILITY OF THE ASSAY FOR ASPIRIN (ASA) AND SALICYLIC ACID (SA) IN PLASMA ($n=5$)

ASA concentration (ng/ml)	Plasma volume extracted (ml)	Coefficient of variation (%)	SA concentration (ng/ml)	Plasma volume extracted (ml)	Coefficient of variation (%)
5	0.5	2.7	50	0.5	2.0
5	1.0	3.0	50	1.0	3.2
50	0.5	2.0	500	0.5	1.8
50	1.0	2.2	500	1.0	2.6
500	0.5	2.4	2000	0.5	3.5
500	1.0	1.1★	2000	1.0	2.5

★$n=4$.

grams from extracts of plasma obtained before and at 30 min and 2 h after an oral 50-mg dose of aspirin in solution taken by a volunteer.

Although 310 nm is not the optimal excitation wavelength for salicylate the relative response for the aspirin peak compared to endogenous peaks was much greater than at 295 nm, and much cleaner chromatograms resulted. Attempts to improve the cleanliness of the extraction by changing the extraction solvent to chloroform or dichloromethane or altering the type or amount of acid used were not successful. The small peak found at the retention time of aspirin was not able to be resolved from aspirin by altering the chromatographic conditions. The peak was equivalent to 1.72 ± 0.92 ng/ml aspirin (mean $\pm$ S.D., $n=10$) and that at the retention time of salicylic acid was equivalent to 6.40 ± 2.27 ng/ml salicylic acid ($n=10$) in plasma samples from people not taking aspirin.

The peak-height response was linear over the concentration range 2 ng/ml to at least 2 μg/ml for aspirin and 20 ng/ml to 5 μg/ml for salicylic acid extracted from 1 ml of plasma. The extraction was $74.6 \pm 4.4\%$ for aspirin and $77.0 \pm 2.4\%$ for salicylic acid from 0.5 ml plasma and $70.2 \pm 3.8\%$ and $66.4 \pm 1.6\%$ for aspirin and salicylic acid, respectively, from 1 ml of plasma, over the entire range (mean $\pm$ S.D., $n \geqslant 12$). The reproducibility of the assay is shown in Table I.

No interfering peaks were found in extracts of plasma from patients taking the medications listed in Table II. Dipyridamole, sulphinpyrazone and the sulphide metabolite of sulphinpyrazone did not interfere at concentrations higher than those achieved therapeutically.

Although it is preferable to use an internal standard, especially in assays involving an extraction, we were unable to find a compound with suitable extraction, detection and chromatographic properties from the extensive list of salicylate derivatives and other acids shown in Table III. The isomer of aspirin, O-acetyl-*m*-hydroxybenzoic acid, which met all these criteria, was unfortunately even more unstable in aqueous solution than aspirin and thus was not considered suitable either. Therefore care must be taken in preparation of the extracts and samples should preferably be assayed in duplicate.

For maximum sensitivity with a post-column reaction system such as this the

TABLE II

DRUGS TAKEN BY PATIENTS WHOSE PLASMA WAS TESTED FOR INTERFERENCE

Amiloride	Frusemide	Prednisolone
Amoxicillin	Glibenclamide	Pilocarpine
Beclomethazone	Heparin	Salbutamol
Carbamazepine	Indomethacin	Temazepam
Coloxyl/Danthron	Insulin	Theophylline
Cimetidine	Metamucil	Thyroxine
Co-trimoxazole	Methyldopa	Tolbutamide
Digoxin	Metoclopramide	Trimethoprim
Ferrous sulphate	Paracetamol	Warfarin
Flurazepam		

reaction conditions and residence time in the coil should enable the reaction to go virtually to completion and at the same time the coil should contribute as little as possible to band spreading. The rate of hydrolysis of aspirin is dependent on the pH of the solution and on the temperature, and increases with increasing pH above 8 and with increasing temperature [12, 13]. Initial estimates of the post-column hydrolysis conditions were determined theoretically after limiting the reaction time to less than 1 min to minimise peak spreading by diffusion, and the temperature to 60°C, just below the boiling point of methanol at atmospheric pressure. Times for the reaction to reach 95% completion at various pH values were then calculated from the hydrolysis rates at those pH values [12] adjusted for temperature using the Arrhenius equation [13, 14] with the average of the activation energies for aspirin hydrolysis found in these two papers. The accuracy of the reaction time estimates is limited firstly by the two-fold difference in activation energy for base hydrolysis found in the two papers and secondly by the pres-

TABLE III

CHEMICAL SUBSTANCES TESTED FOR USE AS INTERNAL STANDARDS

N-Acetyl-5-aminosalicylic acid	2-Hydroxyphenylacetic acid
5-Bromosalicylic acid	4-Hydroxyphenyllactic acid
5-Chloroacetylsalicylic acid	Ibuprofen
5-Chlorosalicylic acid	Indomethacin
5-Chlorosalicyluric acid	Mandelic acid
m-Cresotic acid	2-Methoxybenzoic acid
o-Cresotic acid	5-Methylsalicylic acid
3,5-Dichlorosalicylic acid	Naproxen
Diflunisal	3-Nitrosalicylic acid
Dihydroxybenzenesulphonic acid	5-Nitrosalicylic acid
2,4-Dihydroxybenzoic acid	Pamoic acid
2,6-Dihydroxybenzoic acid	Phenylanthranilic acid
3,5-Dinitrosalicylic acid	Phenylbutazone
Flufenamic acid	3-Phenylsalicylic acid
Frusemide	Tetrafluorosalicylic acid
3-Hydroxybenzoic acid	3-Trifluoromethylsalicylic acid
4-Hydroxybenzoic acid	*p*-Toluic acid
1-Hydroxy-2-naphthoic acid	

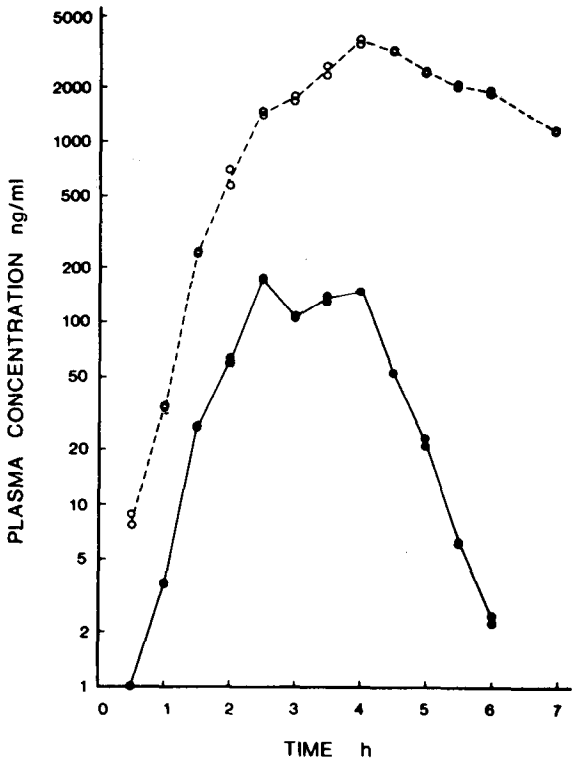

Fig. 2. Plasma aspirin (●) and salicylic acid (○) concentration versus time plots in a volunteer taking 50 mg enteric coated aspirin granules (Astrix) daily. The two symbols at each time point represent duplicate assays of each sample.

ence of a large proportion of methanol in the mobile phase which is likely to alter the rate of hydrolysis. With these initial estimates, the amount of sodium hydroxide to achieve the required pH was calculated, and the sodium hydroxide concentration and the flow-rate of the post-column pump were adjusted accordingly. Under the conditions of this assay (temperature 60 °C, final eluent pH 12–12.5, residence time approximately 40 s) the reaction goes to greater than 95% completion. Experimentally, an increase in strength of the sodium hydroxide solution to1 or 2 *M* causes no further increase in peak height for aspirin despite an increase in pH and thus presumably in hydrolysis rate whereas a decrease in sodium hydroxide concentration to 0.25 *M* caused a 40–50% decrease in peak heights. The band spreading was kept to a minimum by using the finest-bore stainless-steel tubing available and coiling it fairly tightly [15]. Using a UV absorbance detector (280 nm), peak heights for aspirin were found to be 72% of those using the same system without the reaction coil. The coil causes a modest increase in back-pressure of approximately 20–27 bar.

Since the chromatographic separation requires a mobile phase of acidic pH we have used a weak solution of a strong acid, 0.085% phosphoric acid, so that the pH can be easily increased for the hydrolysis step. Because of this a weak buffer

must be used in the back-extraction step in sample preparation. Strong buffers cause solvent effects in the chromatography and give rise to split peaks.

Although we prefer to use physostigmine to minimise aspirin hydrolysis during storage since it is a better plasma esterase inhibitor than potassium fluoride [11], potassium fluoride will not interfere with the sample extraction and chromatography in this assay and may be used if desired.

Plasma aspirin and salicylic acid concentration versus time plots from the pilot pharmacokinetic study are shown in Fig. 2. There was no measurable aspirin or salicylic acid in the pre-dose sample. Enough data points were obtained to estimate the half-life of disappearance of aspirin even from this dose and formulation. The calculated half-life was 20 min.

This assay should allow the further exploration of the possible relationship between plasma aspirin concentrations from low-dose aspirin and effect, using readily available equipment.

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