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Simple method for the determination of 5-aminosalicylic and *N*-acetyl-5-aminosalicylic acid in rectal tissue biopsies

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Abstract

We describe a sensitive high-performance liquid chromatographic method for the determination of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid in rectal tissue biopsies. Samples were derivatised using propionic anhydride and proteins were precipitated with methanol. A Supelcosil ABZ column (150×4.6 mm I.D., 5 μm silica particles) was used with a mobile phase comprising 0.1 *M* acetic acid, acetonitrile and triethylamine (1600:114:6, v/v/v). Fluorescence detection was employed and detection limits were 0.2 ng/mg tissue at a signal-to-noise ratio of three (measured concentration: 5-aminosalicylic acid, 0.254 (0.228–0.286) ng/mg, C.V. 10.7%; *N*-acetyl-5-aminosalicylic acid, 0.18 (0.154–0.198) ng/mg, C.V. 9.8%). This assay was validated for use with serum, urine and faecal samples for which it proved to be both precise and accurate (C.V.<10%, measured concentration within 10%). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: 5-Aminosalicylic acid; *N*-Acetyl-5-aminosalicylic acid

1. Introduction

For many years, sulphasalazine has been a cornerstone in the management of patients with ulcerative colitis [1,2]. Studies of its metabolism and component properties have shown that 5-aminosalicylic acid (mesalazine) is the therapeutically active component, whereas most of the side effects reside in the sulphonamide component [3,4]. This has led to the development of a number of new, colonic delivery 5-aminosalicylic acid formulations and several are in widespread use.

Autoradiographic studies in the rat show that 5-aminosalicylic acid localises within the colonic mucosa [5]. Since systemic 5-aminosalicylic acid levels are low following oral dosing, with both mesalazine and sulphasalazine, the drug is thought to have a predominantly topical mode of action. An understanding of the relationships between mucosal 5-aminosalicylic acid levels, standard pharmacokinetic parameters and clinical efficacy would therefore be of interest.

A mucosal biopsy assay for 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid has been described by De Vos et al. [6]. However, this assay necessitates a time-consuming extraction/evaporation step and has not been validated for serum, urine or faeces. Moreover, the problem of surface contamination of the biopsies with faeces, which also contains drug, was not addressed.

In this paper, we describe a simplified tissue assay

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that is valid for rectal tissue biopsies, serum, urine and faeces. We demonstrate that surface contamination of the tissue biopsy with faeces has a major influence on results and show that there is a poor correlation between tissue drug concentrations and standard pharmacokinetic parameters.

2. Experimental

2.1. Reagents

5-Aminosalicylic acid and 4-aminosalicylic acid were purchased from Sigma (Poole, UK) and Fluka (Buchs, Switzerland), respectively. *N*-Acetyl-5-aminosalicylic acid, propionyl-5-aminosalicylic acid and propionyl-4-aminosalicylic acid (>99% purity) were synthesised by the Department of Organic Chemistry (University of Sheffield). All other reagents were of analytical grade and purchased from Fisher (Loughborough, UK).

2.2. Chromatography system

Chromatographic separations were performed by high-performance liquid chromatography using a Waters (Watford, UK) 510 pump, 717 plus autosampler, 470 fluorescence detector (excitation, 315 nm; emission, 430 nm) and analysed using a 746 data module. A Supelcosil ABZ column (150×4.6 mm I.D., 5 μm silica particles), purchased from Sigma, was protected by a Supelco (Sigma) guard column (20×4.6 mm I.D., 5 μm silica particles). The mobile phase consisted of 0.1 *M* acetic acid, acetonitrile and triethylamine (1600:114:6, v/v/v) at pH 4.3. The flow-rate was 1.5 ml/min, with a resulting pressure of 10.35 MPa, and the analysis was performed at ambient temperature.

2.3. Calibration samples and curves

Stock solutions of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid were prepared by dissolving 20 mg of each compound in 200 ml of water (heated to 70°C). Standard solutions of different concentrations were obtained by serial dilution of the stock solution and each was stored at −80°C. A 4-aminosalicylic acid (4-ASA) solution (60 μg/ml) was prepared for use as the internal standard using a similar protocol. Calibration samples were prepared by adding standard solutions to blank serum, biopsy homogenate, urine, faeces and 0.05 *M* phosphate buffer (pH 7.4). Calibration curves were constructed, using unweighted linear regression, for each analyte from the peak area ratios of the respective analyte to the internal standard versus the amount of analyte. Recovery is expressed as the % response of a processed spiked matrix standard compared to pure standard.

2.4. Sample preparation

Rectal biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Samples were weighed [median 7.1 (3.8–10.9) mg] and then crushed. A 100-μl volume of internal standard solution (15 μg/ml 4-ASA), 400 μl of 0.05 *M* phosphate buffer (pH 7.4), 500 μl of methanol and 20 μl of propionic anhydride were added. Tissue cells were disrupted ultrasonically using a microprobe (Jencons Scientific, Leighton Buzzard, UK) inserted into the suspension for 60 s at 40 W. After vortex-mixing, the samples were left for 30 min at room temperature, to permit protein precipitation, and were then centrifuged at 5000 *g* for 15 min. The supernatant was filtered using 0.5 μm Millex LCR filters (Millipore, Watford, UK) and 20 μl samples were injected directly into the high-performance liquid chromatography (HPLC) system.

Urine was diluted tenfold in 0.05 *M* phosphate buffer (pH 7.4). To a 0.1-ml aliquot, 0.3 ml of 0.05 *M* phosphate buffer, 0.1 ml of internal standard (60 μg/ml 4-ASA), 20 μl of propionic anhydride and 0.5 ml of methanol was added. Following vortex-mixing, samples were allowed to stand for 30 min and were then centrifuged at 5000 *g* for 10 min. The supernatant was filtered and 20 μl were injected directly into the HPLC system.

Faeces were collected directly into 500 ml methanol and mixed thoroughly to prevent acetylation of 5-aminosalicylic acid by faecal bacteria. The collection was weighed and homogenised using a Colworth stomacher (Seward Medical, London, UK). Samples were then prepared using the protocol employed for urinary drug analysis.

Samples of serum (0.5 ml) were added to 0.5 ml of 0.05 *M* phosphate buffer, 50 μl of internal standard (60 μg/ml 4-ASA), 20 μl of propionic

anhydride and 1.5 ml of methanol. After standing for 30 min at room temperature, the mixture was centrifuged at 1500 *g* for 15 min. The supernatant was filtered and 20 μl samples were injected directly into the HPLC system.

2.5. Clinical protocol

2.5.1. Ethical considerations

The studies were approved by the Northern General Hospital Ethics Committee and all subjects gave written informed consent prior to inclusion.

2.5.2. Effects of mucosal washing

Faecal concentrations of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid are considerable following oral dosing with mesalazine and sulphasalazine [7,8]. Surface contamination of the rectal mucosa with faeces is therefore likely to have a major influence on apparent tissue levels when biopsies are taken from unprepared mucosa.

Bowel cleansing with laxatives and enemas may clear surface contamination but leach drug from the tissues, artificially depressing levels. Moreover, accelerating intestinal transit with laxatives may alter the pharmacokinetics of 5-aminosalicylic acid formulations [9]. We therefore chose to take rectal biopsies from unprepared bowel before and after washing the mucosa with a 20 ml jet of 0.9% saline, to assess the effects of surface contamination.

Ten patients with clinically quiescent ulcerative colitis, taking maintenance sulphasalazine or delayed-release mesalazine, and six healthy volunteers, taking delayed-release mesalazine (400 mg three times daily) for a minimum of seven days, were studied. Each subject underwent rigid sigmoidoscopy and rectal biopsies were taken from the anterior rectal wall before and after mucosal washing.

In order to assess the efficacy of the washing technique, three studies were undertaken. Firstly, the lumen–mucosa interface of rectal biopsies from ten healthy volunteers and six patients were examined histologically by both light and fluorescence microscopy. Secondly, we noted during the development of the assay that additional HPLC peaks were apparent, at retention times between 2 and 5 min, when samples were overtly contaminated with faeces. We therefore performed serial dilution experiments on heavily contaminated biopsies to estimate the potential magnitude of faecal contamination. Finally, in vitro studies were performed in which colonic specimens, removed at operation from patients who were not taking mesalazine or sulphasalazine, were pinned, mucosal side uppermost, on cork mats and smeared with faeces containing 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid. Biopsies were taken immediately following smearing, the mucosal surface was then washed in the usual manner with 20 ml of 0.9% saline, and biopsies were repeated.

2.5.3. Variations in tissue 5-aminosalicylic acid and N-acetyl-5-aminosalicylic acid concentrations within the rectum and over time

Six healthy volunteers took delayed-release mesalazine (400 mg three times daily) for 24 days. On days 7, 8, 9, 14 and 21, subjects attended each morning at 09:00 a.m. for rigid sigmoidoscopy. One rectal biopsy was taken from the anterior rectal wall prior to washing and three were taken from separate regions of the rectum after washing. Medication was stopped on day 21 and further rectal biopsies were taken on days 22 and 23. Urine was collected over 24 h on each study day to assess compliance.

2.5.4. Correlations between tissue and serum 5-aminosalicylic acid and N-acetyl-5-aminosalicylic acid concentrations

Eleven healthy volunteers took delayed-release mesalazine (400 mg three times daily) for seven days. On day seven, subjects attended for sigmoidoscopy and rectal biopsy (after mucosal washing) at 09:00 a.m., 12:00, 15:00, 18:00, 21:00 p.m. and at 09:00 a.m. on day eight. Following each sigmoidoscopy, blood samples were drawn from an indwelling intravenous cannula. Urine and faeces were also collected over 24 h.

2.5.5. Statistics

All results are expressed as median (range) unless stated otherwise. The Wilcoxon signed rank test was used to assess intra-subject comparisons and the Mann-Whitney test was used to assess inter-subject comparisons. Correlations were sought using Pearson's product moment coefficient of correlation.

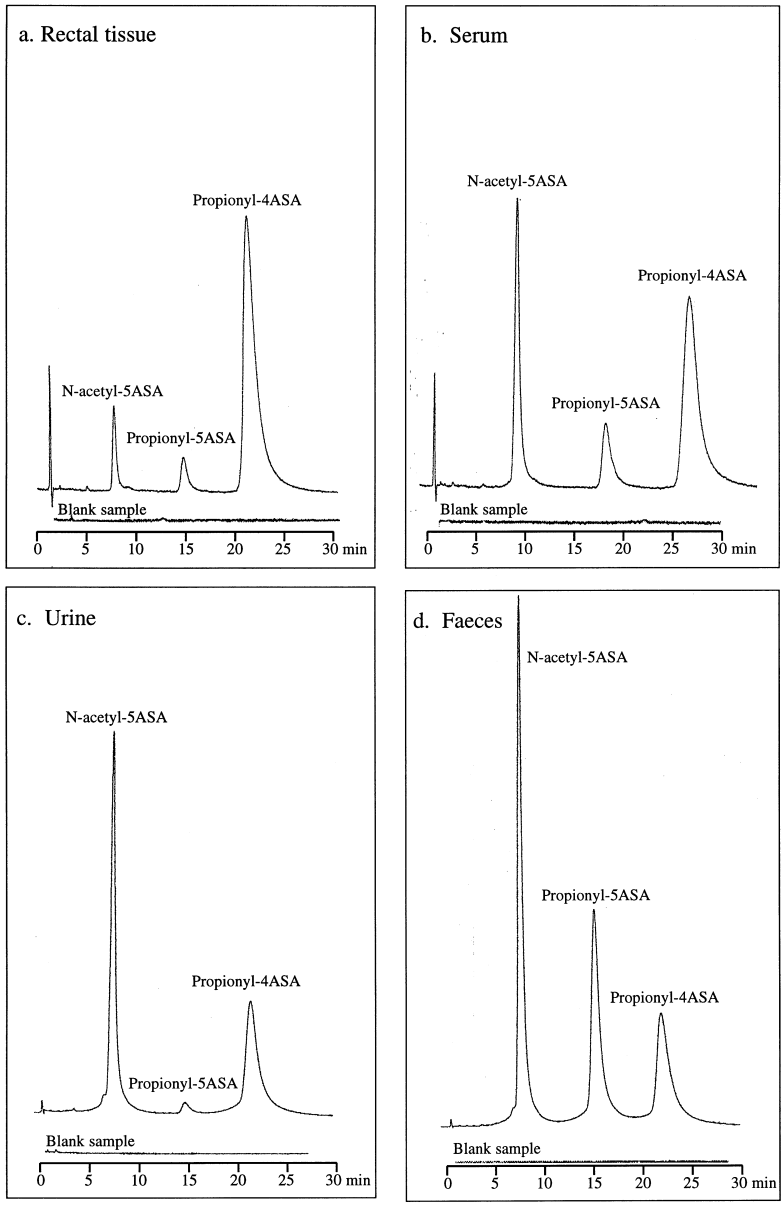

Fig. 1. Typical chromatograms from subjects taking delayed-release mesalazine: (a) rectal tissue biopsy: 5-aminosalicylic acid=1.75 ng/mg, *N*-acetyl-5-aminosalicylic acid=4.31 ng/mg; (b) serum: 5-aminosalicylic acid=331 ng/ml, *N*-acetyl-5-aminosalicylic acid=752 ng/ml; (c) urine: 5-aminosalicylic acid=8 μg/ml, *N*-acetyl-5-aminosalicylic acid=200 μg/ml and (d) faeces: 5-aminosalicylic acid=210 μg/ml, *N*-acetyl-5-aminosalicylic acid=320 μg/ml. Chromatograms from corresponding blank samples are also shown.

3. Results

3.1. Chromatography

Typical chromatograms derived from rectal biopsies, serum, urine and faeces are shown in Fig. 1. Chromatograms derived from subjects taking no medication showed no peaks in the areas of interest. The peaks for *N*-acetyl-5-aminosalicylic acid, propionyl-5-aminosalicylic acid and propionyl-4-aminosalicylic acid (internal standard) were all well resolved.

Correlation coefficients between responses and concentrations in rectal tissue homogenates (0.2–200 ng/mg), measured on five separate days by two different operators, were as follows: 5-aminosalicylic acid, $r=0.9990$ (0.9970–0.9998) and *N*-acetyl-5-aminosalicylic acid, $r=0.9980$ (0.9967–0.9999). The median slopes and their coefficients of variation (C.V.) were: 5-aminosalicylic acid, 333.0 (299.9–357.8), C.V. 7.20%; *N*-acetyl-5-aminosalicylic acid, 398.3 (365.7–411.2), C.V. 4.66%. Residual values (differences between observed and predicted values) were normally distributed (Anderson Darling and Kolmogorov-Smirnov tests: $p<0.01$) and centred around zero (5-aminosalicylic acid=0.63, *N*-acetyl-5-aminosalicylic acid=0.94). The intra- and inter-assay coefficients of variation and recovery over a range of concentrations are shown in Table 1. The lower limits of detection, in rectal tissue samples, at a signal-to-noise ratio of at least three, were 0.2 ng/mg for 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid (100 μl injection). Accuracy and precision derived from five replicate samples at this concentration were: 5-aminosalicylic acid, measured concentration, 0.254 (0.228–0.286) ng/mg, C.V. 10.7%; *N*-acetyl-5-aminosalicylic acid, measured concentration, 0.180 (0.154–0.198) ng/mg, C.V. 9.8%.

Table 1
Inter- and intra-assay coefficients of variation and drug recovery of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid added to biopsy homogenates, serum, urine and faeces

Matrix	Spiked concentration		Measured concentration	Inter-assay C.V. (%)	Intra-assay C.V. (%)	Mean recovery % (SD)
Rectal tissue	*N*-Acetyl-5-ASA	5 ng/mg	5.2 (4.9–5.5)	4.2	3.6	104.7 (4.4)
	5-ASA	5 ng/mg	4.8 (4.5–5.4)	7.2	4.1	97.8 (7.1)
	N-Acetyl-5-ASA	20 ng/mg	20.1 (19.6-22.4)	5.5	4.3	102.6 (5.7)
	5-ASA	20 ng/mg	18.9 (17.9–21.1)	6.7	3.7	96.5 (6.5)
	N-Acetyl-5-ASA	100 ng/mg	99.7 (93.7–100.2)	2.8	1.3	98.3 (2.7)
	5-ASA	100 ng/mg	104.4 (98.3–106.6)	3.3	1.2	102.9 (3.4)
	N-Acetyl-5-ASA	150 ng/mg	144.0 (132.3–147.9)	4.4	2.9	95.2 (4.2)
	5-ASA	150 ng/mg	152.2 (145.1–167.8)	6.3	2.6	103.2 (6.5)
Urine	*N*-Acetyl-5-ASA	100 μg/ml	98.1 (95.4–106.0)	4.6	1.8	100.4 (4.6)
	5-ASA	100 μg/ml	106.3 (100.1–113.0)	5.1	3.1	106.7 (5.4)
	N-Acetyl-5-ASA	250 μg/ml	270.8 (256.5–299.3)	6.1	5.9	108.3 (6.7)
	5-ASA	250 μg/ml	241.2 (227.8–249.2)	3.2	1.6	96 (3.1)
	N-Acetyl-5-ASA	500 μg/ml	487.9 (467.9–514.9)	3.6	2.7	97.9 (3.5)
	5-ASA	500 μg/ml	512.9 (498.7–539.8)	3.1	2.1	102.9 (3.2)
	N-Acetyl-5-ASA	750 μg/ml	734.6 (712.3–769.0)	3.3	1.9	99.2 (3.2)
	5-ASA	750 μg/ml	765.4 (734.5–821.3)	4.2	1.6	102.9 (4.3)
Serum	*N*-Acetyl-5-ASA	100 ng/ml	107.2 (92.3–115.2)	9.1	8.8	106.0 (9.6)
	5-ASA	100 ng/ml	97.8 (91.4–108.2)	6.8	4.2	97.7 (6.6)
	N-Acetyl-5-ASA	250 ng/ml	246.6 (215.7–277.4)	9.1	7	98.6 (9.0)
	5-ASA	250 ng/ml	248.9 (207.5–271.2)	9.7	6.9	97.8 (9.5)
	N-Acetyl-5-ASA	5000 ng/ml	5094.6 (4798.2–5590.2)	5.7	1.9	102.4 (5.8)
	5-ASA	5000 ng/ml	4792.5 (4760.7–5211.3)	3.9	3.9	98 (3.8)
	N-Acetyl-5-ASA	7500 ng/ml	7986.2 (7543.8–8112.1)	2.8	1.9	105.4 (3.0)
	5-ASA	7500 ng/ml	7654.3 (7432.9–7814.1)	1.8	2.7	101.9 (1.8)
Faeces	*N*-Acetyl-5-ASA	100 μg/ml	104.3 (97.3–111.0)	5.4	3.8	103.8 (5.6)
	5-ASA	100 μg/ml	92.5 (90.1–108.2)	7.6	5.3	95.8 (7.3)
	N-Acetyl-5-ASA	250 μg/ml	253.7 (249.4–269.3)	3.9	2.7	102.6 (4.0)
	5-ASA	250 μg/ml	238.5 (230.5–257.3)	4.1	1.1	96.5 (4.0)
	N-Acetyl-5-ASA	500 μg/ml	487.4 (456.3–512.3)	4.4	2.1	97.3 (4.3)
	5-ASA	500 μg/ml	518.7 (487.6–528.9)	3.4	2.7	102.2 (3.5)
	N-Acetyl-5-ASA	750 μg/ml	743.2 (701.2–769.5)	3.4	1.8	98.5 (3.3)
	5-ASA	750 μg/ml	729.3 (700.1–749.2)	3.2	1.2	96.8 (3.1)

All values are expressed as median (range), $n=5$ (unless otherwise stated).
5-ASA, 5-aminosalicylic acid.

Table 2
Accuracy and precision of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid at the limits of detection

Matrix		Spiked concentration	Measured concentration	C.V. (%)
Rectal tissue	*N*-Acetyl-5-ASA	0.2 ng/mg	0.180 (0.154–0.198)	9.8
	5-ASA	0.2 ng/mg	0.254 (0.228–0.286)	10.7
Urine	*N*-Acetyl-5-ASA	0.1 μg/ml	0.100 (0.09–0.107)	5.9
	5-ASA	0.1 μg/ml	0.097 (0.095–0.102)	4.0
Serum	*N*-Acetyl-5-ASA	5 ng/ml	4.669 (3.700–5.079)	7.9
	5-ASA	5 ng/ml	5.070 (4.503–5.700)	5.6
Faeces	*N*-Acetyl-5-ASA	0.1 μg/ml	0.091 (0.086–0.093)	3.0
	5-ASA	0.1 μl/ml	0.090 (0.088–0.964)	3.5

All values are expressed as median (range), $n=5$ (intra-assay).
5-ASA, 5-aminosalicylic acid.

The correlation coefficients derived from five replicate curves, performed on separate days by two different operators, in blank serum (0.005–10 μg/ml), urine (0.1–1000 μg/ml) and faeces (0.1–1000 μg/ml) were: 5-aminosalicylic acid in serum, $r=0.9972$ (0.9921–0.9999), urine, $r=0.9927$ (0.9900–0.9965), faeces, $r=0.9990$ (0.9841–0.9994); *N*-acetyl-5-aminosalicylic acid, serum, $r=0.9973$ (0.9927–0.9993), urine $r=0.9964$ (0.9958–0.9989) and faeces, $r=0.9975$ (0.9929–0.9994). The inter-assay slope coefficients of variation ($n=5$) were: 5-aminosalicylic acid: serum, 7.5%, urine, 4.6%, faeces, 6.5%; *N*-acetyl-5-aminosalicylic acid: serum, 7.1%, urine, 8.5%, faeces, 6.4%. The lower limits of detection, at a signal-to-noise ratio of at least three, for both 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid were 0.1 μg/ml for urine and faeces and 0.005 μg/ml for serum (100 μl injection). Accuracy and intra-assay precision, derived from five replicate samples, at the limits of detection, are shown in Table 2.

The intra- and inter-assay coefficients of variation and recoveries from serum, urine and faeces over a range of concentrations are shown in Table 1. The coefficients of variation did not exceed 10%, further confirming the ruggedness of the assay.

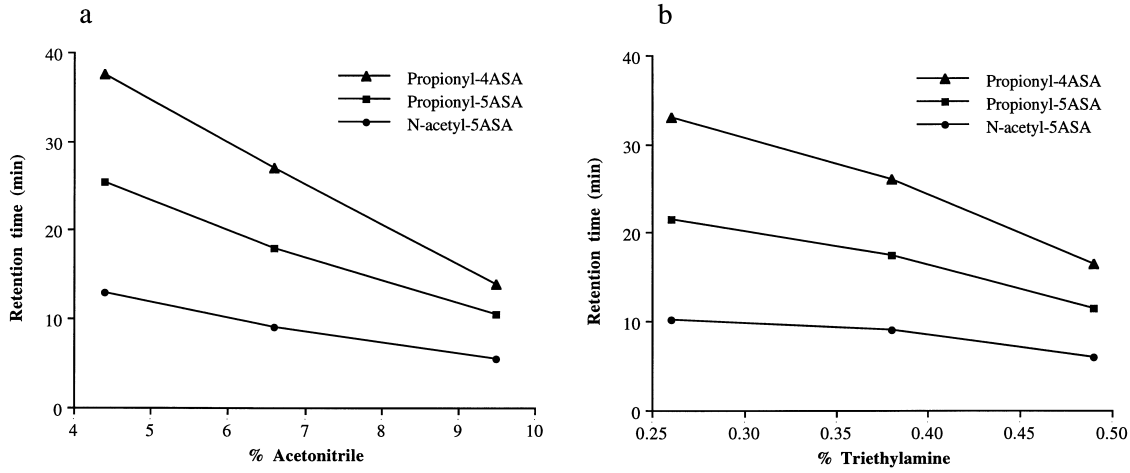

Fig. 2. The influence of the percentage of acetonitrile and triethylamine on retention time.

The effect of varying the percentage of acetonitrile and triethylamine on retention time is shown in Fig. 2.

3.2. Effects of mucosal washing

The effects of washing the rectal mucosa with 20 ml of 0.9% saline on apparent tissue levels of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid are shown in Table 3. In both patients and healthy volunteers, a significant reduction in drug concentration was evident.

Sixteen pairs of rectal biopsies (ten from volunteers, six from patients) were examined by light and fluorescence microscopy before and after washing, to assess the efficacy of washing on surface contamination. Light microscopy revealed no evidence of surface faecal material before or after washing and the epithelial barrier remained morphologically intact after washing. All biopsies showed evidence of tissue fluorescence. Surface fluorescence, however, was seen in only one biopsy before mucosal washing and none after washing.

Chromatograms derived from rectal biopsies overtly contaminated with stool consistently exhibited peaks that were not seen in chromatograms from 'clean' biopsies. Serial dilution studies, however, showed that these peaks became undetectable at dilutions of 1 in 100, whereas tissue drug peaks were often visible at dilutions up to 1 in 10 000.

In vitro studies revealed that mucosal washing with 20 ml of 0.9% saline removed more than 95% of the surface contamination [5-aminosalicylic acid: before washing, 124 (5.6–700) ng/mg, after washing, 0 (0–2.1) ng/mg, $p=0.002$; *N*-acetyl-5-aminosalicylic acid: before washing, 99.6 (5.5–600) ng/mg, after washing, 0.3 (0–7.6) ng/mg, $p=0.002$].

3.3. Variations in tissue 5-aminosalicylic acid and N-acetyl-5-aminosalicylic acid concentrations within the rectum and over time

Rectal tissue concentrations of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid showed modest intrasubject variability but greater inter-subject variability [5-aminosalicylic acid: intra-subject C.V., 54.1 (0–173.2)%, intersubject C.V. 124.9 (88–210)%, $p<0.05$; *N*-acetyl-5-aminosalicylic acid: intrasubject C.V., 34.5 (0–173.2)%, intersubject C.V., 67 (51–182.3)%, $p<0.05$].

Fig. 3a shows the variability in rectal tissue drug concentration and urinary excretion over time. Again intrasubject variability appears less marked than intersubject variability. We were surprised to find, on day 14 of the study, that tissue drug levels fell appreciably in five volunteers. This was associated with a marked decrease in urinary excretion, suggesting poor compliance.

On discontinuing oral mesalazine, tissue levels fell rapidly. After 24 h, tissue *N*-acetyl-5-aminosalicylic acid had fallen from 8.2 (1.9–14.2) to 2.9 (0.3–6.6) ng/mg. Tissue 5-aminosalicylic acid concentrations were virtually undetectable after 24 h (<LOQ–0.3 ng/mg).

Table 3
Effect of washing the rectal mucosa on 'apparent' tissue levels of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid

	Before washing	After washing	
Healthy volunteers (n=6)			
N-Acetyl-5-ASA (ng/mg)	13.9 (<LOQ–324.4)	6.4 (<LOQ–35.9)	$p<0.001$
5-ASA (ng/mg)	2.96 (<LOQ–97.7)	1.1 (<LOQ–20.3)	$p<0.001$
Ulcerative colitis patients (n=10)			
N-Acetyl-5-ASA (ng/mg)	10.3 (0.7–190.8)	4.5 (<LOQ–55.4)	$p<0.05$
5-ASA (ng/mg)	5.5 (<LOQ–245.6)	0.75 (<LOQ–33.2)	$p<0.05$

All values are expressed as median (range).
5-ASA, 5-aminosalicylic acid; LOQ, limit of quantification.

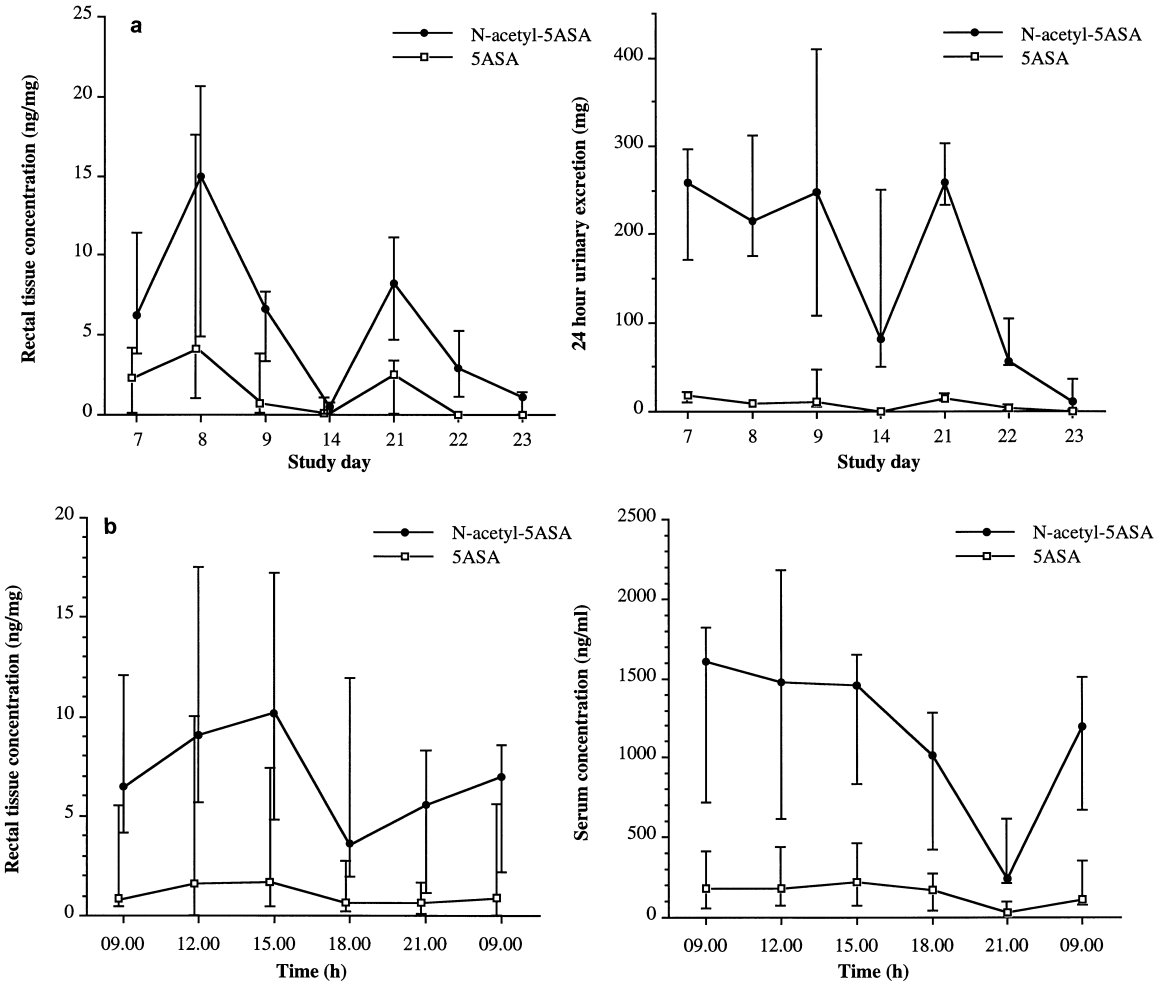

Fig. 3. (a) Rectal tissue drug concentrations and urinary drug excretion over 23 days (median and inter-quartile range). (b) Steady-state rectal tissue and serum drug concentrations over 24 h (median and inter-quartile range).

3.4. Correlations between tissue 5-aminosalicylic acid and N-acetyl-5-aminosalicylic acid concentration and standard pharmacokinetic parameters

Fig. 3b shows the serum and rectal biopsy drug concentrations over a 24-h sampling period in 11 healthy volunteers taking delayed-release mesalazine (400 mg three times daily).

There was no significant correlation between serum and tissue drug concentrations (5-aminosalicylic acid, $r=0.2$; *N*-acetyl-5-aminosalicylic acid, $r=0.28$). Furthermore, no correlations were found between tissue AUC [*N*-acetyl-5-aminosalicylic acid, 167.5 (22–503) ng·h/mg; 5-aminosalicylic acid, 39.4 (7–342) ng·h/mg] and 24 h urinary excretion [*N*-acetyl-5-aminosalicylic acid, 254.3 (57–850) mg, $r=0.0$; 5-aminosalicylic acid, 2.4 (0–161) mg, $r=-0.2$] or faecal excretion [*N*-acetyl-5-aminosalicylic acid, 305 (107–524) mg, $r=0.2$; 5-aminosalicylic acid, 174 (25–527) mg, $r=0.1$].

4. Discussion

Aminosalicylates play an important role in the management of patients with ulcerative colitis [10]. They are of value in patients with active disease and are of particular use in maintaining disease remission. The active component of this group of drugs is thought to act locally, within the colonic mucosa, but its mode of action remains unknown.

Although many have developed HPLC assays for 5-aminosalicylic and *N*-acetyl-5-aminosalicylic acid, most have been limited to one or two biological matrices [11–13]. Several methodologies [14–16] have been developed for use in serum, faeces and urine. De Vos et al. [6] described a tissue biopsy assay but this requires a time-consuming extraction/evaporation step and has not been validated for other biological fluids. A similar assay, described by Palumbo et al. [17] also requires an evaporation step and has only been validated for tissue samples.

In the present study, we have developed a simplified tissue assay that is also valid for serum, urine and faecal samples. This assay utilises propionylation of 5-aminosalicylic acid, as used by Van Hogezand et al. [15], to enhance the relatively poor fluorescence characteristics of 5-aminosalicylic acid. Triethylamine was used by De Vos et al. [6], as an ion-pairing agent, to improve peak symmetry. In our assay, increasing the percentage of triethylamine resulted in a decreased retention time. We also found that increasing the percentage of acetonitrile in the mobile phase decreased the retention time whilst preserving adequate band spacing. In clinical studies, marked variability in drug concentrations was apparent and we employed a longer retention time to preserve band spacing over a wide range of concentrations.

We have also shown that surface contamination of the tissue biopsy with faeces, which also contains drug, may have a significant effect on apparent tissue concentrations. This is not surprising as faecal concentrations of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid are high in patients taking oral mesalazine. Washing the mucosal lining of the rectum prior to biopsy brought about a three-to-five-fold reduction in measured 5-aminosalicylic acid concentrations and a two-fold reduction in *N*-acetyl-5-aminosalicylic acid concentrations. The washing process did not disrupt the epithelial barrier and, since the washing process lasts for little more than 10 s, we feel that it is unlikely to leach significant amounts of drug from the mucosa. Lack of mucosal washing may explain why others have reported considerably higher tissue levels than those found in the present study [18].

We found it difficult to assess the efficacy of mucosal washing in vivo since light and fluorescence microscopy and endogenous HPLC faecal peaks proved insensitive in the detection of faecal contamination. However, our in vitro studies suggest that mucosal washing is highly effective in removing surface contamination.

In the small group of subjects studied, rectal tissue levels of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid showed considerable variability. The cause of this variability is not clear but was more marked between subjects than within an individual. Tissue levels were maintained throughout the day but fell at times of non-compliance and dropped rapidly when the drug was discontinued.

Urinary and faecal concentrations of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid were considerably higher than tissue levels, but no significant correlations were found between serum, urinary and faecal concentrations and tissue drug levels. The relationships between tissue concentrations and clinical efficacy is currently under investigation.

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