

Urinary Excretion Levels of MMX-Mesalazine as a Tool to Assess Non-Adherence

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Abstract

Objective: 5-Amino salicylic acid (5-ASA) is the cornerstone of ulcerative colitis treatment, with (assessment of) non-adherence as a challenge. Multi-matrix release (MMX)-mesalazine has the advantage of once-daily (OD) dosing. Primarily we assessed urinary (NAC-) 5-ASA excretion, as measured by High-Performance Liquid Chromatography (HPLC), in order to monitor nonadherence, in healthy volunteers taking MMX-mesalazine. Secondly, we established urinary (NAC-)5-ASA cut-off levels for (partial) nonadherence.

Method: We studied 25 healthy adult volunteers who used MMX-mesalazine 2400 mg OD (days 1-4), followed by 1200 mg twice daily (BID) (days 8-11), separated by a drug-free interval of 3 days. Daily morning urine spot samples were collected. The cut-off level for adherence was set at the lowest steady state (NAC-)5-ASA urinary concentration level.

Results: Stability of urinary 5-ASA and NAC-5-ASA, stored at room temperature during 24 hours was $96.4 \pm 8.3\%$ and $96.4 \pm 4.1\%$. Recovery of urinary 5-ASA and NAC-5-ASA was $114.3 \pm 10.4\%$ and $107.5 \pm 6.4\%$. The limit of detection and quantification were 1.1 ug/ml and 3.5 ug/ml for NAC-5-ASA and 0.4 ug/ml and 1.3 ug/ml for 5-ASA. The maximal 5-ASA within-run and between-run relative SD were 10.4% and 12.5%. The cut-off level for non-adherence was determined at 9.67 (OD) and at 15.39 (BID) mg/mmol (NAC-) 5-ASA per mmol creatinine.

Conclusion: HPLC is a feasible, sensitive and reproducible method to measure urinary (NAC-) 5-ASA excretion in volunteers taking MMX-mesalazine. This study establishes urinary (NAC-) 5-ASA cut-off levels for MMX-mesalazine non-adherence that may be useful in clinical practice and future trials.

Keywords: Inflammatory bowel disease; IBD; Adherence; Compliance; MMX-mesalazine; High-performance liquid chromatography (HPLC)

Introduction

Adherence is an important element for a successful treatment of patients with ulcerative colitis (UC). 5-Aminosalicylic acid (5-ASA) is a central component in UC treatment, but the reported prevalence of drug non-adherence is high and varies from 40 to 91% [1,5-6].

One of the reasons for this large variation is lack of direct and objective methods to screen for and monitor non-adherence in UC patients. Non-adherence puts patients at an increased risk for relapse [1-6], and steeply increases costs [7]. Already in 2006, a Cochrane review concluded that future trials with 5-ASA should focus on enhancement of patient's adherence [8], but assessing adherence has proven complex. Electronic monitoring using microelectronic chips that log date and time of medication bottle opening, is expensive and does not measure true intake of medication, but only opening of a bottle. On the other hand self-reporting measures are less reliable [9-12]. A more direct and objective way to measure adherence is to assess drug levels in biological fluids such as urine or plasma, and has been reliably measured by High-Performance Liquid Chromatography (HPLC) [13-17]. A potential caveat of drug level monitoring at a clinical visit is the so called 'white coat compliance', which means that adherence tends to improve preceding a clinical visit [11,18,19]. Urinalysis is preferred here because of more stable drug metabolites that reflect medication use over a prolonged period of time. In 2003, Shale reported that 58% of patients with undetectable urinary drug levels admitted to be nonadherent [5]. Subsequent 5-ASA adherence studies using urinalysis applied different formulations, brands, and dosage frequencies. The novel once daily, high dosage (1.2 gr/tablet) MMX-mesalazine formulation has not yet been studied [20-22]. We now report a study to describe feasibility, sensitivity, and reproducibility of High-Performance Liquid

Chromatography (HPLC) to measure urinary (NAC-) 5-ASA excretion in healthy volunteers taking MMX-mesalazine. This allows us to determine cut-off levels for adherence, which can be used in future trials as well as in clinical practice.

Method

Population

This pilot study enrolled 25 healthy volunteers, who were studied for a total of 14 days. Volunteers were recruited via internet or billboard advertisements. Inclusion criteria were age between 18 and 80 years and use of adequate contraceptives during the study period. Pregnancy, significant co-morbidities or the use of co-medication (especially NSAIDs and drugs that possibly effect renal function) were exclusion criteria. After obtaining written informed consent, all volunteers were invited for baseline and screening visits by a research nurse. At baseline we obtained a brief medical history and a physical examination including urine spot and blood samples. Each volunteer received a trial number for identification purposes during the study. All study-related data were documented in a paper case report form (CRF) that was subsequently processed in a computerized database.

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Study design

The schematic outline of the study is described in Figure 1. Volunteers used 2400 mg MMX-mesalazine once daily (OD) (2 tablets of 1200 mg), on study days 1 to 4, and 1200 mg twice daily (BID), on study days 8 to 11. After day 4 and day 11, there was a drug-free wash-out interval of 3 days. OD medication was taken at breakfast (between 7 and 10 AM). BID medication was taken at breakfast (between 7 and 10 AM) and dinner (between 17 and 20 PM). The morning medication was taken under the watchful eye of the research nurse; the evening medication was either taken under nurse supervision, or assisted by webcam or smart-phone. At baseline and at study day 14 a blood sample was collected to analyze creatinine (mmol/l), hemoglobin, leucocytes, platelets, amylase, bilirubine, gamma-GT, and ASAT. The safety profile of MMX-mesalazine is similar to delayed-release mesalazine; it is recommended to check renal function at regular intervals [23-25]. Volunteers recorded adverse events through a purpose designed diary. All volunteers donated daily morning urine spot samples, which were frozen at -20°C, prior to HPLC analysis (samples collected before taking the morning dose of 5-ASA). In a previous study steady state urinary 5-ASA concentrations were reached after 48 hours [5]. Therefore mean individual urinary (NAC-) 5-ASA-excretion was determined on the individual steady state urinary values from day 3 to day 5 and day 10 to 12. The cut-off-level for adherence was defined as the lowest (NAC-) 5-ASA urinary concentration level found in the subjects, taking 2400 mg (OD or BID) MMX-mesalazine. All volunteers received a financial compensation (150 Euro) for their efforts. This trial (ISRCTN15765858) was approved by the Ethics Committee of Radboud university medical center, Nijmegen in the Netherlands.

5-ASA medication

MMX-mesalazine with MMX Multi Matrix System technology

(Mezavant®; Shire Pharmaceuticals) is an oral, high-strength 1200 mg/tablet, OD formulation of 5-ASA, that incorporates a gastro resistant, pH-dependent film coating combined with an MMX Multi Matrix System polymer core. The coating is thought to delay the release of 5-ASA during transit through the upper gastrointestinal tract, while hydrophilic and lipophilic excipients within the MMX tablet core are designed to prolong release of 5-ASA throughout the colon.

High-performance Liquid chromatography (HPLC)

Spot urine samples for further biochemical testing were frozen at -20°C by the volunteers, transferred at room temperature and stored at -20°C in the laboratory until further analysis. No difference in (NAC-)5-ASA concentration was detected after 7 freeze-thawing cycles of urine samples (once freeze thawing: 54.0 mg/mmol creatinine; 7 times freeze thawing 54.1 mg/mmol creatinine). Samples were prepared by solvent extraction and by dilution technique. Routinely, at the start and end of each sequence calibration samples were used. These samples were prepared by adding 4 standard (NAC-) 5-ASA solutions to 0.05 M phosphate buffer (pH 7.4). This was repeated after 42 samples. The reproducibility of our analyses was determined by repeating the test on the same urine sample every 30 samples. Urinary 5-aminosalicylic acid (5-ASA) and N-acetyl-5-ASA (NAC-5-ASA) were measured by HPLC. The total 5-ASA concentration (5-ASA + NAC-5-ASA) is expressed as a ratio relative to the urinary creatinine excretion, to correct for variations in urinary concentration [5]. Urinary creatinine was measured using the Architect C16000 on basis of the Jaffe reaction [26], both obtained from Abbott Diagnostics (Lake Forest, Illinois, USA). Stock solutions of 5-aminosalicylic acid (Cayman Chemical Company, Ann Arbor, Michigan, USA), N-acetyl-5-aminosalicylic acid (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) and 4-aminosalicylic acid (Sigma-Aldrich, St. Louis, Missouri, USA) are

	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
5-ASA taken	-	OD	OD	OD	OD	-	-	-	BID	BID	BID	BID	-	-	-
Urine Sample	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Blood sample	X														X
Research Nurse	X	X	X	X	X				X	X	X	X			X
Diary (AE/SAE)		X	X	X	X	X	X	X	X	X	X	X	X	X	X

(OD = once daily 2400 mg MMX-mesalazine; BID = twice daily 1200 mg MMX-mesalazine)

Figure 1: Schematic outline of the study.

obtained by dissolving each compound in water, with a concentration of 100 µg/ml, 100 µg/ml and 6000 µg/ml, respectively. These stock solutions are stored at -80°C and are used to calibrate and standardize the assay. 5-ASA and NAc- 5-ASA are quantified by HPLC exactly as described by Hussain [13], using a Jasco HPLC system (JASCO, Tokyo, Japan) equipped with a PU-2089 plus pump, an AS-2055 plus auto sampler and a FP-2020 plus fluorescence detector (excitation 315 nm, emission 430 nm). Chromatographic separation of the eluent was attained using a supelcosil ABZ column (150 x 4.6 mm I.D., 5 µm silica particles) protected by a Supelco guard column (20 x 4.6 mm I.D., 5 µm silica particles) both purchased from Sigma. 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. Sample preparation was performed following instruction of Hussain [13], with the exception of filtering the urine after centrifuging. The 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 (4-ASA), 20 µl propionic anhydride and 0.5 ml of methanol was added.

The accuracy was assessed by spiking NAc-5-ASA and 5-ASA in blank matrices. The spiked samples were prepared in at least triplicate at three different concentrations.

The precision is based on the degree of repeatability of an analytical method under normal operation and is expressed as the percent relative standard deviation (RSD). To calculate the RSD 12 samples with known concentrations of NAc-5-ASA and 5-ASA were measured.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the linear regression method. LOD is defined as the lowest concentration of (NAc-) 5-ASA in urine that can be detected. The LOD is expressed as a concentration at 3:1, $LOD=3.3(SD/S)$, where S is the slope of the calibration curves. The limit of quantification (LOQ) is defined as the lowest concentration of (NAc-) 5-ASA in urine that can be determined with acceptable precision and accuracy. The LOQ is expressed as $LOQ=10(SD/S)$. Ten samples were measured to calculate the LOD and LOQ.

Statistics

The sample size of this small intervention study was determined at 25, to the proposal of Julious with regard to pilot studies [27]. We used descriptive statistics for the characteristics of the tested cohort. All results were expressed as mean and SD or median and range values, unless otherwise indicated. Total urinary 5-ASA concentration (5-ASA and NAc-5-ASA) was expressed as a ratio to the urinary creatinine concentration, in mg 5-ASA/mmol creatinine. To determine the cut-off level of adherence as measured by total urinary 5-ASA excretion, we use the individual steady-state urinary 5-ASA-levels, for both dosing frequencies. These individual results are depicted in a scatter plot of urinary total 5-ASA to creatinine ratios. Each point in this plot represents one of the 25 individuals who took part in this study. To describe the reproducibility, variability and the range of urinary 5-ASA-excretion, the median (NAc-) 5-ASA/creatinine values of the total patient group are plotted in a column bar graph.

Results

Demographics

In a 4-month period 30 healthy volunteers were screened to participate in this pilot study. Four volunteers were excluded because of relevant co morbidity and co-medication. One volunteer started with the pilot study, but had to quit on day 6, because of arthritis of the

shoulder that necessitated NSAID treatment, which was an exclusion criterion. In total twenty-five volunteers were included and completed the whole study period of 14 days. Demographic variables of this study group and adverse events are presented in Table 1. Mean age of the volunteers was 38.1 years (SD 8.5); fifty two percent of the volunteers were male. Adherence was 100% assessed by nurse controlled medication intake.

HPLC

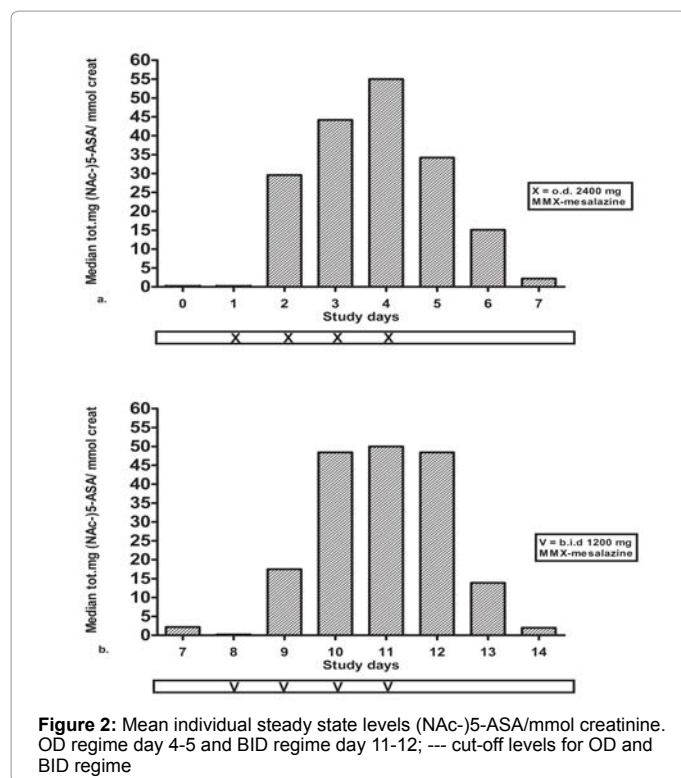
Stability, accuracy, sensitivity and specificity

Stability of 5-ASA and NAc-5-ASA in urine, stored at room temperature during 24 hours was $96.4 \pm 8.3\%$ and $96.3 \pm 4.1\%$, respectively. Stability data of urinary (NAc-) 5-ASA concentration at room temperature up to 192 hours are depicted in a Supplementary file (Figure 5a and 5b). No effect of 7 freeze-thawing cycles was detected on the concentrations of 5-ASA and NAc-5-ASA. The retention time for respectively NAc-5ASA, 5 ASA and 4-ASA is 6, 11 and 16 min. The accuracy or recovery of urinary 5-ASA and NAc-5-ASA was respectively $114.3 \pm 10.4\%$ and $107.5 \pm 6.4\%$. The linearity of the method was determined through analysis of five calibration curves containing non-zero concentrations.

The limit of detection (LOD) was 1.16 µg/ml for NAc-5-ASA and 0.43 µg/ml for 5-ASA. The limit of quantification (LOQ) was respectively 3.51 µg/ml (NAc-5-ASA) and 1.29 µg/ml (5-ASA). The highest measured within-run relative standard deviation (R.S.D.) was respectively 4.1% for NAc-5-ASA, and 10.4% for 5-ASA. The highest measured between-run R.S.D was 9.4% for NAc-5-ASA and 12.5% for 5-ASA.

Variable	N (%)
Volunteers (N)	25 (100)
Male (N, %)	13 (52)
Medical history	
- None	7 (28)
- Minor irrelevant surgery (appendectomy/tonsillectomy, knee surgery)	12 (48)
- IBS	
- Various	1 (4)
	5 (20)
Severe adverse events	0 (0)
Adverse events (AE)	17 (68)
- Renal	0 (0)
- Gastrointestinal	
- Miscellaneous	3 (12)
	14 (56)
AEs related to study medication	0 (0)
AEs not related to study medication	5 (20)
AEs probably related to study medication	12 (48)
Variable	Mean (SD)
Age (yrs)	38.1 (8.5)
BMI	25.6 (6)

Table 1: Demographic variables and adverse events.



Cut off level 5-ASA

In Figure 3 the median (Nac-)5-ASA levels expressed as a ratio to the urinary creatinine concentration in the total patient group (N=25) are depicted. Steady state levels were respectively reached at day 4 and day 11, and lasted until day 5 and day 12. Figure 4 shows mean individual steady state levels measured at day 4 to 5, and at day 11 to 12. Cut-off level for adherence was defined as the lowest individual steady state (Nac-)5-ASA urinary concentration level found in the studied subjects. In the once daily medication schedule the cut-off level for adherence was 9.67 mg (Nac-)5-ASA per mmol creatinine, and in the twice daily group this cut-off was determined at 15.39 mg (Nac-)5-ASA per mmol creatinine. Figure 1 shows individual data of the total patient group on day 6 (48 hours after last medication taken) and day 7 (72 hours after last medication taken). This figure provides insight into (partial) adherence of patients in daily practice, as the measured (Nac-)5-ASA urinary concentration level at day 6 is comparable to the value measured in a patient not taking his/her medication for two consecutive days. The lowest individual (Nac-)5-ASA urinary concentration level measured on day 6 is 0.03 mg (Nac-)5-ASA per mmol creatinine (median 15.1; range 0.03-66.24), and on day 7 this is 0.02 mg (Nac-)5-ASA per mmol creatinine (median 2.2; range 40.61).

Adverse events

During the pilot study no serious adverse events occurred. Adverse events probably related to the study medication occurred in 48% of the total group, and 12% complained of gastrointestinal symptoms (3/25). Routine laboratory measurements, particularly creatinine, remained unchanged during the complete study period (Table 2).

Discussion

This study documents the pharmacokinetics of a novel

mesalazine formulation (MMX). We found that the use of HPLC for 5-ASA urinalysis in volunteers using MMX-mesalazine is feasible, reproducible, stable and sensitive, and is accompanied by a low inter- and intra-assay variation. This is in line with previously published studies, with other 5-ASA formulations, which showed that HPLC is a reliable tool to recover 5-ASA metabolites from urine samples [13-17].

There has been a paucity of pharmacokinetic data on MMX-mesalazine. A literature search revealed published data derived from a single phase II clinical trial, the manufacturer-prescribing information, and an abstract published in 2007 [27-30]. These sources document a high pharmacokinetic variability among volunteers, comparable to our data in Figure 3 and 4. They achieved steady state after 2 days, in contrast to our study (steady state achieved after 3 days in both dosing frequencies). We determined objective 5-ASA urinary cut-off values for (partial) non-adherence in our study that may be used in clinical practice or for the purpose of determination of adherence in clinical trials. Medication adherence occurs when the patient takes his/her medication according to the prescribed dosage, time, and frequency. Logically, if patients take no medication at all, they are absolute non-adherent, which corresponds to undetectable 5-ASA urinary levels. In practice, however, most patients occasionally forget to take their medication [17,31], which defines them as partially non adherent. As such, it is important to provide objective 5-ASA urinary cut off values to define partial non-adherence.

A strong element of our study is the use of one single 5-ASA formulation, and the directly observed intake of MMX-mesalazine, which is efficacious in attaining complete adherence. A comprehensive review on this topic concluded that urinary 5-ASA excretion is comparable for many oral 5-ASA formulations, but that 5-ASA preparations with a foregut release profile demonstrate greater variability in urinary excretions. More recently released 5-ASA formulations, as MMX-mesalazine, were not included in this review [21].

Recent literature mentions a distinct variability in 5-ASA metabolism and distribution following oral dosing, and discovered an association between higher doses of oral mesalazine with higher serum concentrations and urinary excretion [32], a finding that was described before [5]. We defined cut off values for non-adherence for two different dosage schedules, which we consider an asset. We found a higher cut off level in the twice daily group (15.39 mg/mmol (Nac-)5-ASA per mmol creatinine) compared to the once daily group (9.67 mg/mmol (Nac-)5-ASA per mmol creatinine). Significance levels could not be determined because of the small sample size. Even though dosing frequency does not affect steady-state pharmacokinetics of delayed-release mesalazine, earlier studies found peak levels of 5-ASA serum concentrations in the early morning following divided daily dosing [32-34]. The indiscriminate use of different formulations, and dosage schedules may provide inaccurate values in defining a cut-off value in urinary 5-ASA excretion.

This study comes with some limitations. First of all, it is a small intervention study that carries the design of a pilot study. Secondly, we studied healthy volunteers instead of UC patients. A substantial part of the UC patients have co-morbidity or co-medication that may affect the pharmacokinetic properties of 5-ASA [30,35]. In patients with active UC, pharmacokinetics might be influenced by diarrhea, changed intestinal transit time, and luminal PH. The contribution of these elements and the ultimate effect on 5-ASA pharmacokinetics is incompletely understood and the literature reports conflicting results [21,36-40]. Interestingly, the pharmacokinetics of healthy volunteers

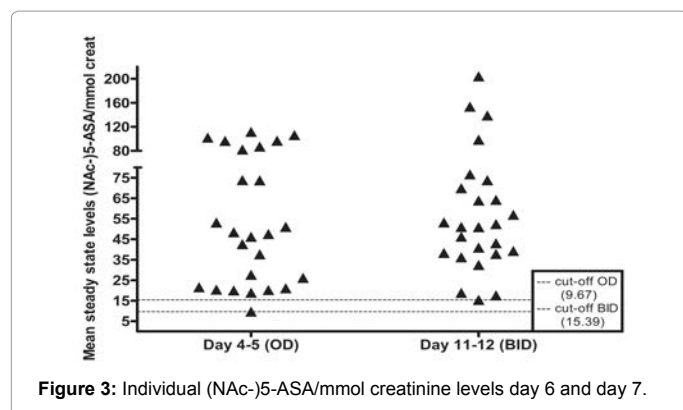


Figure 3: Individual (Nac)-5-ASA/mmol creatinine levels day 6 and day 7.

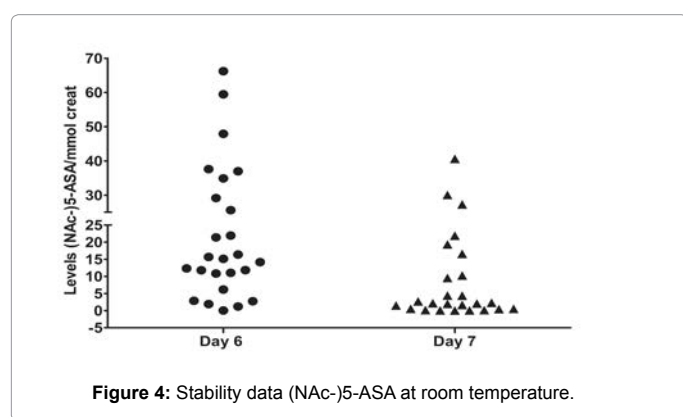


Figure 4: Stability data (Nac)-5-ASA at room temperature.

Variable	Visit day 1 (mean, SD)	Visit day 14 (mean, SD)	p-value
Creatinine (Umol/l)	74.6 (6.4)	75.4 (9.1)	NS
Amylase (U/l)	64.6 (19.3)	67.4 (27.4)	NS
Hemoglobin (mmol/l)	8.9 (0.6)	9.0 (0.7)	NS
Thrombocytes (*10 ⁹ /l)	244 (50.7)	260 (61.1)	NS
Leucocytes (*10 ⁹ /l)	6.7 (1.8)	6.7 (2.0)	NS
Bilirubine (Umol/l)	10.6 (4.0)	10.6 (4.4)	NS
Gamma-GT (Umol/l)	27.6 (15.0)	28.5 (16.4)	NS
ASAT (U/l)	24.4 (4.7)	27.1 (8.6)	NS

Table 2: Laboratory results visit day 1 and 14.

and patients with quiescent UC are comparable [40,41], and similar tissue concentrations of 5ASA and Nac-5ASA have been detected in both groups [13]. Therefore, in this specific subgroup of UC, data might be extrapolated. We did not perform formal plasma, C_{max} analysis or AUC, which are accepted methods in pharmacokinetic studies. We specifically wanted to describe feasibility and reproducibility of HPLC in case of MMX-mesalazine users, and chose spot urinalysis because of the applicability of this method in daily practice. Of course, white coat compliance might affect results of spot urinalysis in the outpatient clinic. But, as described before [5,42], and again shown in our study, it took more than three days without medication at all, to find near undetectable 5-ASA urine levels. For these reasons, we think this effect is negligible. Another matter of concern can be the costs of HPLC, which of course must be seen in the light of the costs of non-adherence.

In summary, HPLC spot 5-ASA urinalysis is fast, reproducible and sensitive. The cut off values for non-adherence in once or twice daily dosing are slightly different. Relevant for clinical practice is that we describe objective cut off values for (partial) non-adherence, which is

the most common way of non-adherence. A spot 5-ASA urine sample below the described cut off values should trigger the treating physician to consider and discuss possible non-adherence.

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