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Methylene blue MMX® tablets for chromoendoscopy. Bioavailability, colon staining and safety in healthy volunteers undergoing a full colonoscopy

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ABSTRACT

Methylene blue-MMX® tablets are proposed as an aid for detection and visualisation of adenomas and carcinomas in patients undergoing colonoscopy, by improving their detection rate and highlighting the presence of the intestinal dysplastic lesions.

Single total doses of 100 and 200 mg were administered to healthy volunteers undergoing a bowel cleansing preparation and a full colonoscopy to investigate the colonic staining. The pharmacokinetics of methylene blue and the safety after exposure to the tablets were also investigated.

With 200 mg, the best staining, assessed as the sum of *acceptable* and *good* staining, was achieved in the ascending colon and rectosigmoid (75% subjects each), the transverse and the descending colon (approximately 63% each). *Absence of staining* or *overstaining* were reported for no colonic region of interest in any subject. Similar results were observed in the 100 mg dose group.

Methylene blue blood concentrations reached a peak (C_{max}) in a median time (T_{max}) of 12 h with 100 mg and 16 h with 200 mg. AUC_{0-t} was 10.7 ± 6.7 μg/mLxh after 100 mg and 25.2 ± 7.4 μg/mLxh after 200 mg. Half-life ranged between 9 and 22 h after the lower dose and between 6 and 26 h after the higher dose. The cumulative urinary excretion was about 28% after 100 mg and about 39% after 200 mg up to 60 h post-dose.

The overall frequency of adverse events after single dose of the test product administered along with a bowel cleansing preparation was 39%, but only one was related to the test product: abnormal transaminases. The most frequent adverse event was a transient polyuria (17%). One serious adverse event (gastrointestinal haemorrhage) led the subject to study discontinuation and hospitalisation and another subject withdrew the study due to one adverse event (haematemesis). Either event was not related to methylene blue.

1. Introduction

Methylene blue is able to enhance the detection of dysplastic lesions in the colon by increasing the contrast at mucosal level between normal and altered tissue without impairing the tissue vital functions.

Methylene blue MMX® tablets are a new oral modified release formulation manufactured using a multimatrix structure (MMX®, Cosmo Technologies Ltd., Ireland). MMX is a modified release technology that ensures a colonic drug release through the targeted choice of the formulation core and coating components. The MMX tablets combine the active substance with excipients different in affinity for aqueous solvents (hydrophilicity or hydrophobicity) which comprise a gastro-resistant coating. These characteristics allow the tablets to arrive unaltered to the terminal ileum as previously shown by the gastrointestinal transit and colonic delivery demonstrated for other MMX formulations by pharmaco-scintigraphic investigations in healthy male volunteers [1][2]. Upon dissolution of the gastro-resistant coating by increased pH approximately from the terminal ileum onwards, the tablets release the active ingredient in proximity to the mucosa in an extended way while they travel throughout the colon towards the rectum.

The absolute bioavailability of methylene blue was investigated in a previous Phase I study of Methylene blue MMX® 200 mg tablets after single oral doses of 200 and 400 mg in healthy volunteers after the intake of 2 L of bowel cleansing preparation in 2 h [3]. The previous study formulation was a monolithic tablet, with a drug unitary content, different from that used in the present study. Systemic exposure to methylene blue was shown in all subjects with concentrations

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increasing for 12 h. The peak was reached in a median of 16 h. A complete absolute bioavailability of methylene blue calculated as ratio between AUC_{0-t} oral/iv corrected for the dose was shown (F_{abs} = 139.19 ± 52.00%).

Methylene blue has the property to be selectively absorbed into the cytoplasm of actively absorbing cells such as the intestinal columnar epithelium and to stain the epithelium of small intestine and colon upon simple exposition [2–4] [5–7]. Therefore, it has been often used to screen for colonic neoplasia [8], to diagnose villous atrophy and to screen for areas of dysplasia and carcinoma [2, 8, 9]. Methylene blue evidences the superficial structure of lesions thanks to the different degree of active mucosal stain uptake and highlights the contrast and the differences between cell types [10]. In the gastrointestinal epithelium the dysplastic areas and cancerous lesions generally present a different absorption of methylene blue [4]. Optical magnification endoscopy and chromoendoscopy have gained renewed interest as means for the early detection of minute lesions in patients referred for colonic cancer screening or surveillance [9, 11]. Chromoendoscopy is a technique in which methylene blue highlights specific structural changes in the mucosa that would otherwise not be evident [4, 9]. Methylene Blue MMX® tablets aim at the diagnostic detection of irregular foci or contrast enhanced areas in colon and rectum during the screening or surveillance endoscopy through the modified colonic release of the vital stain. The self-administration of the methylene blue tablets during the bowel cleansing preparation converts the conventional procedure performed by the endoscopist spraying the dye directly on suspected areas into a novel chromoendoscopic procedure. The tablets were investigated also in specific clinical Phase II and III trials [12–15]. The aims of the Phase II trials were (i) to evaluate and verify the colonic mucosal staining efficacy of Methylene Blue MMX® 25 mg modified release tablets when administered to patients undergoing full colonoscopy with different administration schedules during the intake or at the end of the bowel cleansing preparation administered the day before the procedure, (ii) the efficacy of tested tablets in the detection of intraepithelial neoplasiae in patients with inactive ulcerative colitis with a diagnosis of ≥8 years and (iii) the preliminary efficacy of the tested tablets in terms of detection of polyps in patients undergoing routine colonoscopy and receiving the bowel preparation according to a full dose or a split dose regimen.

In a Phase III, multi-centre, placebo-controlled, randomised, double-blind, parallel group study, methylene blue tablets showed a significantly higher adenoma detection rate [12] than the current standard of care technology, namely the high definition colonoscopy.

The present study, that is part of the clinical product development, aimed at preliminarily investigating the staining efficacy, the pharmacokinetic profile and the safety profile of methylene blue administered as MMX tablets at the doses of 100 and 200 mg to 24 healthy male volunteers who underwent a standard 4-lL polyethyleneglycol (PEG)-based bowel cleansing preparation and a full colonoscopy.

2. Methods

2.1. Study design

The present study was a single ascending dose, open-label, safety, bioavailability and efficacy study. Primary objective was the investigation of the staining efficacy in the totality of regions and in the single colonic districts after total oral doses of 100 and 200 mg of methylene blue administered to healthy volunteers undergoing a standardised bowel cleansing preparation the day before a full colonoscopy. The study was also aimed at investigating the kinetic profile of methylene blue in whole blood and urine and the safety and the tolerability of the tablets. The study was designed in 2 parts consisting in one period each. Subjects enrolled for the 1st part received a total oral dose of 100 mg. In the 2nd part, a 2nd cohort of volunteers was enrolled and administered a total oral dose of 200 mg. The blood sampling and the urine collection schedule were planned up to 60 h post-dose considering the previously published kinetic data [1].

The doses were chosen on the basis of the results of the previous clinical trial in healthy subjects, who received 200 and 400 mg of methylene blue [1].

The decreased strength of the individual tablet (25 mg) in comparison to the one used in the previous clinical study (a monolithic tablet containing 200 mg of methylene blue) allowed to administer, at each time, lower doses of the compound (25, 50 or 75 mg), with the aim to obtain a better and more homogeneous colon distribution and staining fractioning the total dose and to lower the risk of an ineffective staining due to unwanted tablet expulsion.

The frequency of tablets administration was selected based on the results obtained in previous Phase II colon staining efficacy studies [13–15].

According to the literature, the most frequent untoward effect of methylene blue was the effect specifically wanted, that is the temporary blue staining of tissues. Less frequent untoward effects were diarrhoea, dysuria and headache. In the previous study [1], only non-serious adverse events occurred. Related events occurred to 8/22 subjects. Most of the events were mild and transient. Abnormal transaminases, gastrointestinal disorders and dysuria frequency was 13.6%. According to this previous experience, the safety assessments of the present study were focused on the recording of adverse events and on the clinical laboratory assays. It is also known that untoward effects may be exacerbated in case of glucose-6-phosphate dehydrogenase deficiency. Therefore, the enzyme was assayed specifically in the present study.

2.1.1. Study population and criteria for inclusion

The study was performed at the Phase I Unit of CROSS Research S.A., Arzo, Switzerland.

Healthy males were included into the trial according to the following main inclusion criteria: (i) age of 18 to 65 y, (ii) a BW ≥60 kg, (iii) good health based on medical history, physical examination, a 12-lead electrocardiogram (ECG) and routine haematology and blood chemistry tests, (iv) use of highly effective contraceptive methods for at least 2 months prior to the study start [19], (v) willingness to provide written informed consent.

Main exclusion criteria were the standard criteria for bioavailability estimation of new drugs, namely (i) intake of any medication, (ii) a history of drug, caffeine (> 5 cups coffee/tea/day) or tobacco (≥10 cigarettes/day) abuse, (iii) history of alcohol consumption in excess of two drinks per day, as defined by the U.S.D.A. dietary guidelines [20].

The study was descriptive and non comparative. Therefore, the sample size was not derived from a statistical power calculation. A total of 24 subjects were planned to be included in the study.

2.1.2. Investigational treatments and dose regimen

Besides the investigational product Methylene blue MMX® 25 mg tablets manufactured by Cosmo S.p.A. Italy, Selg-Esse® 1000 laxative solution by Promefarm S.r.l., Italy, was also used to prepare the subjects for colonoscopy. In the 1st study part, a total oral dose of 100 mg (4 tablets) of methylene blue 25 mg tablets was to be administered, as follows:

- tablet 1 after the intake of the 2nd litre of bowel preparation;
- tablet 2 after the intake of the 3rd litre of bowel preparation;
- tablets 3 and 4 after the intake of the 4th (and last) litre of bowel preparation.

In the 2nd study part, a total oral dose of 200 mg (8 tablets) of methylene blue 25 mg tablets was administered, as follows:

- tablets 1 and 2 after the intake of the 2nd litre of bowel preparation;
- tablet 3, 4 and 5 after the intake of the 3rd litre of bowel preparation;

➢ tablets 6, 7 and 8 after the intake of the 4th (and last) litre of bowel preparation.

Methylene blue tablets were swallowed without crashing or chewing with 200 mL of still water.

All the volunteers received a full dose of the bowel cleansing preparation, starting from 17:00 for the first subject, with an interval of 45 min between consecutive subjects. The subjects had been fasting for at least 4 h before bowel cleansing preparation intake. The volunteers drank at least 250 mL of the preparation every 15 min, so that the intake of the cleansing preparation was completed within 4 h; afterwards, the subjects drank 1 additional litre of still mineral water in a time interval of 30–60 min.

During the intake of laxative the subjects were recommended to drink additional still mineral water.

On study day 2, before undergoing colonoscopy, all the subjects were anaesthetised using a propofol 1% injection. The individual dose was adjusted depending on the subject's body weight and sedation status, considering that the recommended dose is 4–12 mg/kg/h. Subjects were confined in the Phase I Unit during the whole study periods.

2.2. Ethical procedures

The study CRO-PK-11-249 – Sponsor code CB-17-01/02 was approved by the independent ethics committee of Canton Ticino on 22FEB11. Ref. nr. 2409. The Swiss Federal Health Authorities (Swissmedic) authorised the study on 09NOV11 and assigned the reference number 2011DR1176. The study was conducted in compliance with the Swiss ordinance on clinical trials of therapeutic agents and in accordance with the Declaration of Helsinki and the general principles of ICH Harmonised Tripartite Guidelines for GCP. Subjects did not undergo any study procedure before signing the written informed consent form. The first subject was enrolled on 28MAR12 and the last subject completed the trial on 11MAY12.

2.3. Efficacy variables and data analysis

The gastroenterologist inspected the study subject's colon by colonoscopy, using a thin (about 14 mm in diameter) flexible tube connected to an Olympus device for colonoscopy. During colonoscopy, the subject laid on a bed in semi-prone position, with the left arm straight alongside and the right arm flexed in the front. After the exam, the subject was monitored in a recovery area and discharged 4 h after the end of colonoscopy.

The efficacy of methylene blue staining of the colon was evaluated scoring it for each single considered region of interest: ascending colon, transverse colon, descending colon and sigmoid/rectum. The total staining score was calculated as the sum of the scores of all the 4 colon areas.

The staining scores were defined as follows: 0 = no staining, 1 = traces of the staining (poor traces in colon mucosa), 2 = detectable staining (at least the 25% of colon mucosa was stained), 3 = acceptable staining (at least the 50% of colon mucosa was stained), 4 = good staining (at least the 75% of colon mucosa was stained) and 5 = over stained (100% of colon mucosa was over stained).

2.4. Pharmacokinetics variables and data analysis

The following PK parameters were measured and/or calculated for methylene blue according to a non-compartmental model using the validated software WinNonLin® 5.2 (Pharsight Corporation):

C_{max}	Maximum observed blood concentration
T_{max}	Time to achieve C_{max} from the time of administration of the last methylene blue tablet
$t_{1/2}$	Half-life, calculated, as $\ln 2/\lambda_z$.where λ_z is the terminal rate constant
AUC_{0-t}	Area under the whole blood methylene blue concentration-time curve from the time of administration of the last methylene blue tablet to the last observed concentration time t, calculated by the linear trapezoidal rule
$AUC_{0-\infty}$	Area under the concentration/time curve extrapolated to infinity, calculated, as $AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable drug concentration
AUC_{extra} (%)	Percentage of the residual area (C_t/λ_z) extrapolated to infinity in relation to the total $AUC_{0-\infty}$, calculated, if feasible, as $100 \times [(C_t/\lambda_z)/AUC_{0-\infty}]$
Xu_{0-t}	Amount of methylene blue excreted in urine from the time of administration of the last methylene blue tablet to the last observation time t
$\%Xu_{0-t}$	Percentage of methylene blue excreted in urine from the time of administration of the last methylene blue tablet to the last observation time t

2.5. Sample collection, handling and analytics

The concentration of methylene blue was determined in whole blood at the following times before and after administration of the last methylene blue tablet:

➢ pre-dose (0), 4, 5, 6, 7, 8, 9, 10, 12, 16, 20, 24, 36, 48 and 60 h post-dose;

and in urine in the following time intervals before and after the administration of the last methylene blue tablet:

➢ pre-dose (0), 0–4, 4–8, 8–16, 16–24, 24–48 and 48–60 h post-dose.

Blood samples for PK analysis were collected using an indwelling catheter with switch valve and transferred in about 30 into EDTA blood collection tubes, mixed and divided into two glass tubes (aliquots B1 and B2), stored protected from light on ice for no > 20 min and stored frozen at $\leq -70\,^{\circ}C$ until analysis.

During each interval, urine was collected into containers, kept refrigerated at $\approx 4\,^{\circ}C$ and protected from light. Total urine volumes were measured and after thorough mixing, two aliquots (U1 and U2) of 10 mL each were stored protected from light in glass tubes at $\leq$-70 °C pending analysis.

The concentration of methylene blue in whole blood and urine was determined at Analytisch Biochemisch Laboratorium ABL BV, the Netherlands, using fully validated LC–MS/MS methods with a lower limit of quantification (LLOQ) of 20 ng/mL for blood and 50 ng/mL for urine. Long term stability tests indicated that methylene blue stored frozen at $\leq -70\,^{\circ}C$ is stable up to 92 days in whole blood and up to 71 days in urine. The two LC–MS/MS methods for the determination of methylene blue in human EDTA whole blood and human urine samples produced accurate and precise results. The same validated method used in the previous study [1] was applied.

2.6. Safety variables

The safety variables included:

➢ the recording of adverse events during the whole study duration
➢ blood pressure, heart rate and body weight measurements at

Table 1
Mean (± SD) baseline demographic data.

Treatment	Safety analysis set	Age	Height	BW
Males	Treated subjects	(y)	(cm)	(kg)
100 mg	5 (100%)	40.6 ± 2.3	178.0 ± 5.1	80.5 ± 6.4
200 mg	18 (100%)	33.7 ± 8.4	178.4 ± 5.6	79.7 ± 9.4

BW: Body Weight.

screening, pre-dose and upon discharge
- physical examinations, ECG and routine blood chemistry, coagulation, haematology and urinalysis laboratory assays performed at screening and upon discharge after each dosing.

3. Results

3.1. Disposition of subjects

Twenty-four (24) male Caucasians, aged 21 to 47 y were enrolled as planned. Baseline demographic data are summarised in Table 1.

Five (5) out of 6 subjects enrolled in the 1st study part received the treatment and completed the blood and urine sampling for the pharmacokinetic analysis. The colon staining efficacy evaluations could be performed for only 3 of them.

All 18 subjects enrolled in the 2nd study part received the planned treatment, but one subject discontinued the study due to safety reasons before undergoing the blood and urine sampling and the colonoscopy. Another subject completed both the urine sampling and the colonoscopy, but discontinued the study due to safety reasons before collecting the last 2 blood samples.

3.2. Methylene blue blood concentration after single oral dose

After single dose administration of either 100 or 200 mg the blood methylene blue vs. time profiles were as in Fig. 1 in linear scale. The main kinetic whole blood parameters measured or calculated for both doses are shown in Table 2.

After single doses of 100 and 200 mg, the drug substance became quantifiable in blood after 4–8 h and 4–12 h after administration of the last methylene blue tablet, due to the peculiar modified release profile of MMX tablets. Systemic exposure to the active ingredient reached a peak in a median time of 16 h after 200 mg and of 12 h after 100 mg. Peak blood concentration increased proportionally with the dose. On average, C_{max} and AUC were twice higher after the 200 mg dose than after the 100 mg dose. Elimination half-life ranged between 9 and 22 h after 100 mg and between 6 and 26 h after the higher dose.

3.3. Methylene blue urinary excretion after single oral

The main kinetic urine parameters of methylene blue are described in Table 3.

The totality of the subjects had quantifiable excreted amounts of methylene blue in the 8–60 h post-dose interval after both 100 and 200 mg.

3.4. Colon staining

Frequency of subjects receiving 200 mg of methylene blue for each score and region of interest is presented in the Fig. 2. Number of subjects for each score is also reported.

The most frequent score in all regions was "*acceptable*" (score 3: ≥ 50% stained area). Score 4, i.e. "*good*" (≥ 75% stained area) was the second most frequently reported.

On the basis of the sum of the frequency of scores 3 (acceptable) and 4 (good), best staining was achieved in the rectosigmoid and the ascending colon (75% subjects each), followed by the descending colon and transverse colon (approximately 63% each).

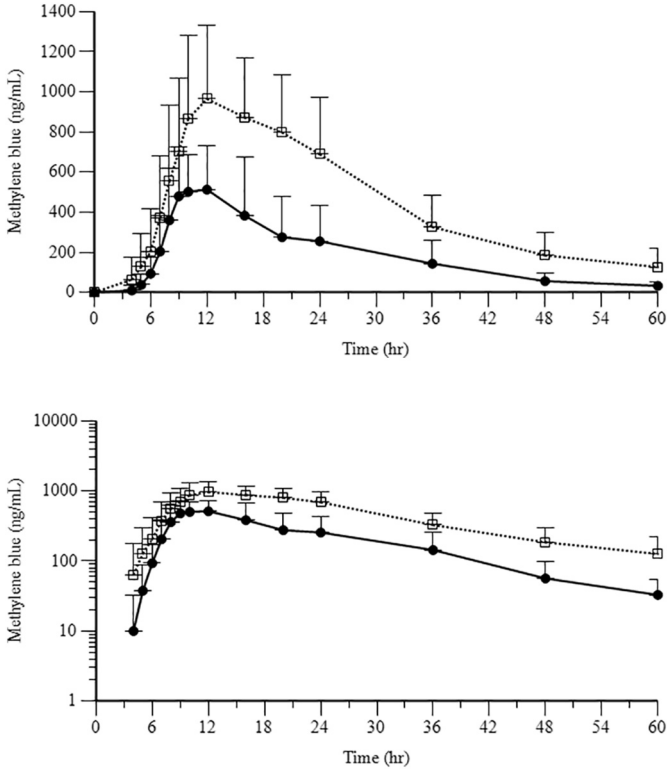

Fig. 1. Mean (± SD) blood methylene blue concentrations (ng/mL) vs. time profiles after single dose administration of 200 mg (□ dotted line) or 100 mg of Methylene blue MMX® tablets (● solid line). Linear (top) and semilogarithmic (bottom) scale.

For the subjects receiving the 100 mg dose, the sum of frequency of scores 3 (*acceptable*) and 4 (*good*) was achieved in the rectosigmoid (100% subjects), followed by the ascending colon (66.7% subjects, score 3 only).

Frequency of subjects with a satisfactory staining quality, defined as subjects with a total score ≥ 8 and no score 5 (i.e. over-staining of the colonic mucosa) was 100% in both dose groups. Indeed, the total score was above 8 and single scores were < 5 for all subjects in both treatment groups.

3.5. Safety

Adverse events occurred to 8/18 subjects (44.4%) in the 200 mg group and to 1/5 subjects (20%) in the 100 mg group. The majority of reported adverse events was not judged to be in relationship with the treatment.

Only one adverse event was judged as related to methylene blue: a mild increase in alanine aminotransferase. The related event occurred to 1/18 subjects (5.6%). No follow-up could be performed because the subject did not return to the Phase I Unit after the end of the study (lost to follow-up). The reported AEs are summarised by system organ class and preferred term in Table 4.

The most frequent reaction was polyuria which occurred at a frequency of 22.2% (4/18 subjects) and occurred only in the 200 mg group.

One subject experienced haemorrhagic anaemia which was classified as a serious adverse event, required hospitalisation and was unrelated to methylene blue. On the day after colonoscopy, the subject started to suffer from haemorrhagic anaemia which worsened one day

Table 2
Mean (± SD) of main PK parameters of blood methylene blue after single dose administration of either 100 or 200 mg of methylene blue. For T_{max} median and range are reported.

Treatment	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0\text{-}t}$ (ng/mLxh)	$AUC_{0\text{-}\infty}$ (ng/mL × h)	$T_{1/2}$ (h)
100 mg	573.60 ± 175.83	12.00 (9.00–16.00)	10720.98 ± 6676.52	11531.46 ± 6599.10	13.87 ± 5.09
200 mg	1149.12 ± 261.95	16.00 (10.00–24.00)	25161.72 ± 7417.20	28558.72 ± 9755.20	15.08 ± 5.85

C_{max}: Maximum observed blood concentration; T_{max}: Time to achieve C_{max}; $AUC_{0\text{-}t}$: Area under the concentration curve from administration to the last observed concentration time t; $AUC_{0\text{-}\infty}$: Area under the concentration/time curve extrapolated to infinity; $t_{1/2}$: Half-life.

Table 3
Mean (± SD) cumulative excretion of methylene blue after single dose administration of either 100 or 200 mg.

Treatment	$XU_{0\text{-}t}$ (mg)	$XU_{0\text{-}t}$ (%)
100 mg	28.02 ± 11.71	28.02 ± 11.71
200 mg	77.34 ± 31.61	38.67 ± 15.80

Xu: Cumulative urinary excretion.

later and was renamed as gastrointestinal haemorrhage. During hospitalisation, a pre-existing Mallory-Weiss syndrome was diagnosed through oesophago-gastro-duodenoscopy.

Another subject suffered from nausea during the bowel cleansing preparation. Later, the event worsened and the subject experienced one episode of haematemesis which was unrelated to methylene blue. Afterwards, the subject discontinued the study without undergoing the colonoscopy and the blood and urine sampling for kinetics.

4. Discussion

Methylene blue administered orally as MMX tablets at the doses of 100 and 200 mg along with a full dose bowel cleansing preparation and before a colonoscopy was absorbed from the colonic epithelium and became available systemically (appearance in the blood) between 4 and 12 h post-dose. These data are consistent with the known peculiar delivery mechanism of MMX tablets which start to progressively release the methylene blue when the intestinal pH is ≥7, i.e. approximately from the terminal ileum onwards. Similar intestinal transit times were observed also for other MMX formulations in previous pharmaco-scintigraphic investigations in healthy men and are in agreement with less

Table 4
Display of adverse events. Number of subjects reporting adverse events by system organ class and low level term.

System organ class	100 mg – $N = 5$	200 mg – $N = 18$
Preferred term	N subjects	N subjects
All adverse events	1	8
Gastrointestinal disorders	0	4
Abdominal discomfort	0	2
Haematemesis	0	2
Gastrointestinal haemorrhage	0	1
Melaena	0	1
Nausea	0	1
Vomiting	0	1
Urinary tract signs and symptoms	0	4
Polyuria	0	4
Nervous system disorders	1	1
Headache	1	0
Migraine	0	1
Blood and lymphatic system disorders	0	1
Anaemia	0	1
Haemorrhagic anaemia	0	1
General disorders and administration site conditions	0	1
Fatigue	0	1
Investigations	0	1
Alanine aminotransferase increased	0	1
Vascular disorders	0	1
Orthostatic hypotension	0	1

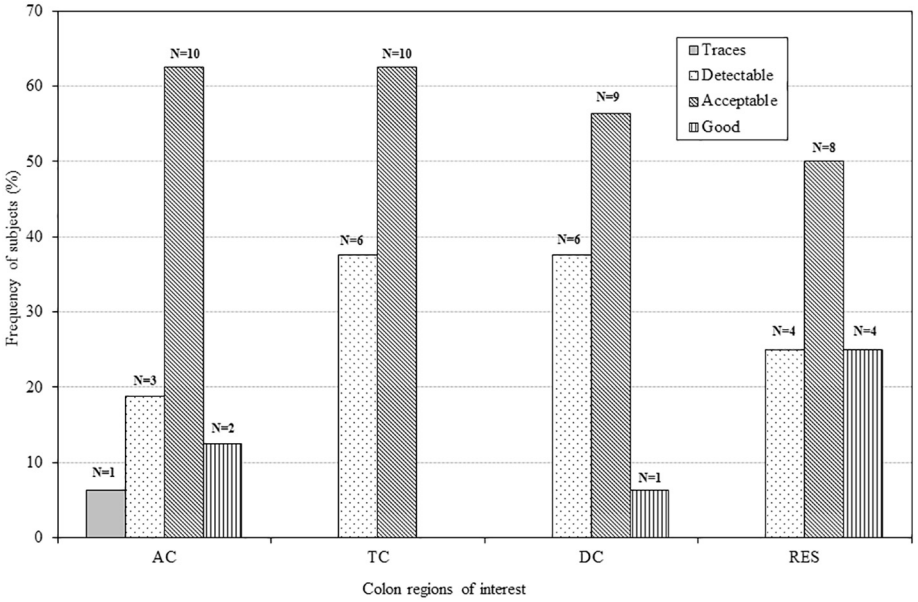

Fig. 2. Frequency of subjects (%) for each staining score in each colon region of interest. 200 mg dose group. AC = Ascending colon; TC = Transverse colon; DC = Descending colon; RES = Rectosigmoid. $N = 16$.

recent literature [21]. In the previous pharmaco-scintigraphic studies, MMX tablets were detected in the ascending colon between 6 and > 24 h in one study and reached the ileo-caecal region in 3.3 ± 1 h in the other one, while Davis et al. showed that the mean transit time through the small intestine is about 3 to 4 h on average and that it does not vary with the physical conditions of the subject or the size of the dosage form or the presence of food [21]. In conclusion, the time of appearance of methylene blue in the blood is consistent with a mainly colonic absorption.

After appearance in blood, methylene blue increased post-dose and attained a concentration peak in a median time of 16 h after 200 mg and 12 h after 100 mg. Time to peak ranged 10–24 h in the subjects' cohort after the dose of 200 mg. This kinetic profile in the absorption phase of the dye ensures an appropriate duration to the mucosal staining and, assuming that the initial blood appearance is indicative of the start of mucosal staining, it suits the timing of a full colonoscopy scheduled for the day after the tablet intake. The endoscopy should be ideally scheduled from 12 to 16 h after administration of the last methylene blue tablet in order to be performed at optimal conditions of colonic staining. However, both methylene blue AUC observed in the study (25.2 ± 7.4 μg/mLxh after 200 mg) and absolute bioavailability known from the previous study [1] evidence a complete absorption of the substance dose, with a ramping concentration starting from 4 h post-dose, and presenting a sustained level up to 36 h post-dose. Assuming that systemically quantifiable levels of methylene blue mirror the mucosal staining and the contrast enhancement, a colonoscopy performed between 8 and 24 h post-dose could grant the maximal effect of enhanced lesion detection.

Within the study dose range, methylene blue absorption and kinetics showed linearity.

The cumulative excretion of methylene blue showed consistency with the results observed in the previous study [1], where after single i.v. injection the percentage was about 40% of the injected dose. In the present study, after 200 mg, the cumulative excretion accounted for 38.67% which is very close to the result of the previous study after the same oral dose (39.67%). After 100 mg, the cumulative excretion was 28.02% of the administered dose. The remaining 60% of the administered dose, which was not excreted in urine, may include also demethylated metabolites of methylene blue, e.g. azure B, which were not assayed in the present study.

The pharmacokinetic observations support also the efficacy results.

With respect to the quality of the staining with the dose of 200 mg, it was scored as satisfactory in the totality of the evaluated subjects. In fact, for all subjects the sum of all scores in the four colonic regions was above 8 and the majority of the subjects had a mucosal staining *acceptable* to *good*. In addition, neither over-staining of the mucosa (score 5) nor absence of staining were detected in any region for any subject. Similar results were observed in the 100 mg dose group. However, the sample size in the 100 mg group was not sufficient to draw conclusions on the staining efficacy for the lower dose, which was not considered as effective as the 200 mg dose in the following Phase II and III studies.

After intake of methylene blue throughout the study, the frequency of adverse events after intake of 200 mg of methylene blue was 44.4%, which is consistent with the overall frequency of adverse events observed in the previous study [1]. Only one related AE occurred to one out of 18 subjects receiving 200 mg of methylene blue, namely an abnormal value of alanine aminotransferase. This observation was consistent with those of the previous study [1].

In conclusion, the safety results confirmed the favourable tolerability profile of the tablets.

The results of the study demonstrated that methylene blue is released in the colonic tract of the bowel, becomes systemically bioavailable to a high extent and is well tolerated by healthy subjects when given as MMX® tablets along with 4 lL of a PEG-based bowel cleansing preparation before a full colonoscopy. Study results showed that methylene blue tablets at a total dose of either 100 or 200 mg had no impact on the subjects' bowel cleansing and preparation for the endoscopy. According to the dose regimen applied in the present study, the intake of methylene blue tablets was scheduled for the evening before the day of colonoscopy during and at the end of the intake of the laxative. The colonic release of methylene blue was not affected by the previous or concomitant transit of the laxative, since the appearance of the dye in the blood stream observed between 4 and 12 h after the dose was consistent with the results of the previous study [1].

Study results confirmed the preliminary knowledge about the safety, tolerability and pharmacokinetics of methylene blue in healthy subjects collected in the previous Phase I study and gave evidence to the suitability of the intake of Methylene blue MMX® tablets concomitantly to the intake of the bowel cleansing preparation and before a full colonoscopy. The present study results gave also preliminary evidence of the efficacy of methylene blue MMX tablets in terms of colonic mucosa staining and contrast enhancing which was further investigated as supportive of the intestinal lesion detection rate, as evidenced in the further clinical development studies.

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The Sponsor reviewed and approved the study design, was informed about the collection of data, reviewed and approved the analysis and the interpretation of data, reviewed and approved the manuscript for publication.

MMR reviewed and approved the design of the study, was responsible for the clinical activities and collected the data, AV drafted the study protocol and co-ordinated the study, AF performed the full colonoscopies and took part in the clinical activities, AR evaluated the colonic staining, AFDD drafted the manuscript.

All authors read and approved the manuscript.

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