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Research paper

Cation exchange resins as pharmaceutical carriers for methylene blue: Binding and release

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Abstract

Methylene blue is a competitive inhibitor of the glutathione reductase of *Plasmodium falciparum* and is used in combination with other antimalarial drugs leading to a renaissance of methylene blue in malaria therapy. Its bitter flavour and tissue colouring property impair compliance, especially in children. These problems may be solved by binding the cationic methylene blue to cation exchange materials as pharmaceutical carriers in order to mask the undesirable properties. However, such carriers are only useful if the antimalarial is released under physiological conditions. The binding to seven cation exchange resins was studied. Ion exchangers on acrylic or methacrylic acid basis bound between 1.54 and 2.16 g methylene blue chloride trihydrate per gram ion exchanger. Polymers on divinylbenzene or styrene basis with sulphonic acid groups bound 306 and 384 mg of methylene blue chloride trihydrate per gram ion exchanger. In aqueous solution at pH of 1.5, nearly all bound methylene blue was released. The release of methylene blue from (meth)acrylic acid polymers in the presence of proteins and fat was not affected. From these data ion exchangers present a promising group of pharmaceutical carrier for the safe and compliant drug administration of methylene blue to children.

Keywords: Malaria; Methylene blue; Cation exchange resins; Binding; Release

1. Introduction

The use of the thiazine dye methylene blue for the treatment of malaria has been described more than a century ago [1,2]. The mechanism of its antimalarial activity is still not fully understood, but methylene blue has been shown to inhibit glutathione reductase of *Plasmodium falciparum* [3] thus decreasing intracellular levels of reduced glutathione, which are essential for the degradation of haematin [4,5]. Impaired degradation might therefore promote accumulation of haematin, which is toxic to the parasites. Antimalarial activity of methylene blue alone and in combination with other antimalarials has been confirmed in vitro [6] and compared with other thiazine dyes methylene blue had the highest activity against different isolates of *P. falciparum* with IC_{50} values in the low nanomolar range [7]. After oral administration of 100 mg methylene blue [8], a standard dose for treatment of methemoglobinemia [9], whole blood concentrations of up to 100 nmol/l (=28.4 ng/ml) were reached in healthy individuals. On the basis of the in vitro results this dosage is therefore expected to confer antimalarial effectiveness.

For dose finding, pharmacokinetic evaluation, and assessment of the safety of methylene blue a series of clinical studies was recently performed [10–13]. These studies revealed that therapeutic doses of methylene blue can safely be administered to glucose-6-phosphate dehydroge-

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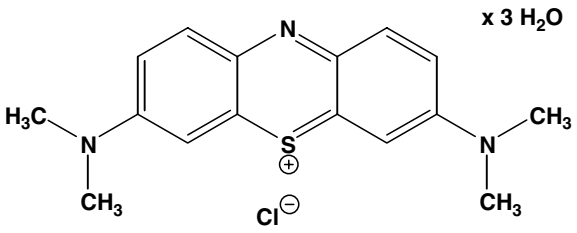

Fig. 1. Chemical structure of methylene blue chloride·3H_2O.

nase deficient adults [12] and to young children with malaria [13]. The most important challenges were the almost unbearable taste of methylene blue, leading to vomiting especially in children, and its tissue colouring properties, which may last for several days after ingestion.

Methylene blue includes a cationic sulphur atom with chloride as a counterion (Fig. 1), which can be substituted by carboxylic or sulphonic acid groups of ion exchange resins. We aimed to quantify methylene blue binding to different ion exchangers as a basis for the development of a palatable pharmaceutical formulation suitable to mask its taste and colouring properties. A further condition was preferential and complete release of the compound at pH values as low as expected in the stomach.

2. Materials and methods

2.1. Chemicals

Methylene blue chloride trihydrate ($C_{16}H_{18}N_3SCl\cdot 3H_2O$; CAS-No. 7220-79-3) ME141 from Spectrum Lab Products (Gardena, California/USA) was used throughout all experiments and fulfilled the requirements of USP/NF.

The cation exchange resins were Indion 234, Indion 234S, Indion 254, and Indion 294 from Indion Resins (Ankleshwar, India) and Amberlite IRP 64, Amberlite IRP 69, and Amberlite IRP 88 from Rohm and Haas Francs S.A.S. (Paris, France) fulfilling the requirements of USP/NF. Indion 234 and Indion 234S differ in particle size. Amberlite IRP 88 and Amberlite IRP 64 are structurally identical, only differing in the counterion (IRP 64 is protonated). Additionally Amberlite IRP 88 is structurally comparable to Indion 294, but both are from different manufacturers. Chemical and physical properties of the ion exchangers, including their characteristics with respect to structure, particle size, and counterions are summarised in Table 1.

Water was deionised and filtered by an HP 6UV/UF TKA system (Niederelbert, Germany). For acidification 0.5 M hydrochloric acid and for alkalinisation 0.1 M sodium hydroxide from Merck KGaA (Darmstadt, Germany) was used. The release of methylene blue in presence of fat and proteins was studied using common fresh milk (3.5% fat content) and a commercial product called Heilnahrung HN25 (powdered baby food containing 66.4 g carbohydrates, 12.8 g fat, and 12.8 g protein per 100 g) from Milupa (Friedrichsdorf, Germany).

2.2. Equipment

Binding and release experiments were performed in double-walled glass flasks (100 ml) tempered to 20–60 °C using a thermostat FP50HD from Julabo (Seelbach, Germany). The resulting temperature difference between tempering liquid and sample liquid was below 1 °C. To prevent adsorption only Teflon coated stirring bars were used for stirring the aqueous methylene blue/ion exchanger suspension (stirrer velocity ~1500 rpm). Measurements of pH in

Table 1
Ion exchangers (IE), parameter, binding, and release of methylene blue chloride trihydrate (MB) at room temperature

Ion exchanger (IE)	Indion 234	Indion 234S	Indion 254	Indion 294	Amberlite IRP 64	Amberlite IRP 69	Amberlite IRP 88
Structure/ matrix	Crosslinked polyacrylic acid with carboxylic groups	Crosslinked polyacrylic acid with carboxylic groups	Divinylbenzene/ styrene with sulphonic groups	Crosslinked polyacrylic acid with carboxylic groups	Methacrylic acid/divinyl-benzene	Divinylbenzene/ styrene with sulphonic groups	Methacrylic acid/ divinyl-benzene
Particle size [mm]	⩽0.15	⩽0.075	⩽0.15	⩽0.15	No specification	No specification	No specification
Counterion	K^+	K^+	Na^+	K^+	H^+	Na^+	K^+
Max. binding*	2162	2086	384	1630	223	306	1542
SD [mg MB/g IE]	86	166	101	82	72	39	15
Release at pH 1.5*	2161	n.d.	n.d.	1615	n.d.	n.d.	1410
SD [mg MB/g IE]	156			151			14
Mass of 1 g loaded IE*	2644	n.d.	n.d.	2229	n.d.	n.d.	2073
SD [mg]	119			115			10

* Mean and standard deviation of $n = 3$ independent experiments; n.d. = not determined

aqueous suspensions were performed with a Knick Calimatic 765 pH meter (Berlin, Germany).

Falcon and Eppendorf tubes were vortexed by a Julabo Paramix II mixer (Seelbach, Germany) at maximum velocity (~3600 rpm). Eppendorf tubes (1.5 ml) were centrifuged in an Eppendorf centrifuge 5415R (Wesseling-Berzdorf, Germany) and 50 ml Falcon tubes in a Jouan GR422 (Fernwald, Germany) centrifuge.

Quantification of methylene blue in solution was performed with the UV/VIS-photometer Labsystems Multiscan RC Type 351 and the corresponding software Ascent Version 2.4.2 from Thermo Scientific (Waltham, MA, USA).

2.3. Preparation of solutions, calibration, and quality control samples

For binding experiments methylene blue solutions (1%, 100 ml) were prepared by weighing 1.00 g methylene blue chloride trihydrate into a 100 ml double walled flask. 50 ml deionised water was added. After stirring for 15–30 min additional 50 ml deionised water was added and the solution was stirred for further 15 min.

For desorption experiments higher amounts of methylene blue maximum loaded ion exchangers were required. Therefore methylene blue solutions (1%, 1000 ml) were produced by adding 10.00 g methylene blue chloride trihydrate to 250 ml intensively stirred deionised water. After 30 min, additional 750 ml deionised water was added and the solution was stirred for a further 30 min.

Separate calibration and quality control (QC) samples were prepared based on separate weighings of methylene blue reference compound in deionised water. Concentrations of calibration samples were 10.1, 20.2, 40.4, 50.5, 80.8, and 101 μg/ml, and QC samples had concentrations of 30.0, 50.0, and 90.0 μg/ml.

2.4. Sample preparation and quantification of methylene blue in aqueous solution

Unknown samples taken from binding and release experiments were transferred to 1.5 ml Eppendorf tubes and centrifuged for 30 s at 16,100*g*. The supernatant was transferred into another tube and diluted 1:10 or 1:100 with deionised water to reach the calibration range. Aliquots (100 μl) were then transferred onto a 96-well plate and analysed. The concentration of methylene blue chloride trihydrate in aqueous solution was determined by UV/VIS photometry at the working wavelength of 600 nm. Each 96-well plate corresponds to one analytical batch which contained six calibration solutions (100 μl) in the range of 10.1–101 μg/ml, six QC samples (30.0, 50.0, and 90.0 μg/ml in double determination), and different numbers of unknown samples.

2.5. Analytical method validation

The analytical procedure was validated according to the recommendations of the FDA Guidance for Industry [14]. Therefore three analytical batches each with QC samples at the lower, medium, and higher area of the calibration range (30.0, 50.0, and 90.0 μg/ml; $n = 6$ each) and six QC samples at the lower limit of quantification (10.1 μg/ml) were analysed. Accuracy was calculated on the basis of the quotient of the averaged QC measurements and the nominal value and expressed in percent. Precision was defined as the ratio of standard deviation and mean calculated value in percent. These values were calculated within-batch and batch-to-batch. Additionally pH-dependent changes in analytical accuracy were determined at pH 2.9 and 10.8.

2.6. Binding of methylene blue to ion exchange resins

In double walled glass flasks 0.25 g Indion 234, 0.25 g Indion 234S, 0.50 g Indion 294, 0.50 g Amberlite IRP 88, 1.00 g Indion 254, 1.00 g Amberlite IRP 64, or 1.00 g Amberlite IRP 69 were added to 100 ml vigorously stirred methylene blue solution (1%). Samples (1 ml) were drawn after 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min to quantify methylene blue remaining in solution. This procedure was repeated three times for each ion exchange resin at room temperature. Additionally each ion exchanger was tested at 40 and 60 °C and with decreased (pH ~2.0) and increased pH (pH >8.0). After binding of methylene blue to ion exchangers they were washed with deionised water until the methylene blue concentration in the washing water was below the lower limit of quantification. Subsequently the loaded ion exchangers were dried over silica gel and weighed to confirm the mass balance.

Higher quantities of ion exchange resins (Indion 234, Indion 294, and Amberlite IRP 88) were loaded using an up-scaled procedure with 1000 ml of methylene blue solution (1%) for desorption experiments. Two batches were produced for the ion exchangers Indion 234 (4.02 and 4.32 g), Indion 294 (6.01 and 6.03 g), and Amberlite IRP 88 (6.26 and 6.25 g) by stirring the suspension for 120 min, transferring to 50 ml Falcon tubes, and centrifugation at 3000*g* for 10 min. The supernatant was disposed and the sediment washed with deionised water until methylene blue concentration in the supernatant was below the lower limit of quantification. The loaded ion exchangers were dried over silica gel to achieve uniform and constant material for the desorption experiments.

2.7. Release of methylene blue from ion exchange resins

In double walled glass flasks loaded ion exchanger, equivalent to 1 g methylene blue chloride trihydrate, was added to 100 ml deionised water and stirred at room temperature until a homogeneous suspension was reached. Subsequently the pH was decreased under continued stirring by dropping in 0.5 M hydrochloric acid. The pH value was measured continuously and at stable pH values of 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, and 1.0 (equilibration time ~ 10 min)

samples were taken and methylene blue concentrations in the solution were measured. The influence of temperature on methylene blue release was evaluated at 37 °C and room temperature. To assess the influence of fat and protein on the release of methylene blue deionised water was replaced by fresh milk (3.5% fat) or Milupa Heilnahrung HN25 (15.6 g HN25 filled up to 100 ml with fresh water resulting in 2.0% fat and 2.0% protein). All experiments were repeated threefold.

2.8. Calculations and statistical methods

Calibration curves were determined for methylene blue chloride trihydrate using the respective calibration samples. Therefore UV absorbance of methylene blue in solution was measured and linear regressions were performed for each analytical batch using standard procedures of the Ascent software.

Amounts of methylene blue bound to or released from ion exchangers were calculated with Microsoft Excel 2003 and data are expressed as mean values ± standard deviation (SD) using the molecular weight of methylene blue chloride trihydrate (373.9 Da). However, when bound to ion exchangers the methylene blue cation (284.4 Da) is bound without chloride and water. Therefore the weight of free/dissolved methylene blue cation is 24% lower. For direct comparison of adsorbed and released compound the results are always expressed as methylene blue chloride trihydrate.

3. Results and discussion

3.1. Analytical method validation

In deionised water the quantification of methylene blue by UV/VIS-photometry was linear between 10.1 and 101 μg/ml. During validation the mean linear correlation coefficient was $R = 0.994(\pm 0.003)$. All deviations of QC samples, lower limit of quantification, and upper limit of quantification compared to the theoretical value (accuracy) were <10% within-batch and batch-to-batch. The relative standard deviation (precision) of all quality control samples, lower limit of quantification, and upper limit of quantification were <6% within-batch and batch-to-batch. The influence of pH on quantification of methylene blue was negligible. Modifying the pH of QC samples from neutral to 2.9 or 10.8 resulted in accuracy deviations of +0.9% and −4.2%, respectively. Because a sample dilution of 1:100 with pure water was usually required in the release experiments no substantial change in pH was expected. To quantify methylene blue in milk or Heilnahrung HN25, no further sample treatment was needed, because proteins and fats precipitated by acidification and were separated by centrifugation. During validation no substantial adsorption of methylene blue to materials was detected and washing of loaded ion exchangers resulted in a loss below 1.0%.

3.2. Preliminary annotations and experiments

Methylene blue chloride trihydrate lost 5.8% of its weight during the drying procedure in an exsiccator over silica gel, which may correspond to one crystal water. When stored at room temperature and humidity for more than 24 h, the weight rose by ~2.0%. In deionised water methylene blue (1%) already formed acidic solutions (pH 3.8), however when ion exchangers were added pH values were nearly neutral (pH 6–7).

The unloaded ion exchangers adsorbed up to 17% water within hours if exposed to room humidity. During storage in closed containers, no weight gain was found. Due to its protonation, IRP 64 suspended in water is acting as a weak acid (pH 5.3) and as expected all other ion exchangers are acting as bases (pH 8.4–9.4). By acidification (e.g. by hydrochloric acid), protonation of the cation exchangers is expected replacing the former counterion (Na^+, K^+, or methylene blue cation). In preliminary experiments loading capacity and saturation limit of the ion exchangers were determined. Sorption experiments in 100 ml deionised water were started with the ratio of 1:1 (m/m) of ion exchanger to methylene blue chloride trihydrate. Because of complete decolouration, the amounts of Amberlite IRP 88 and Indion 294 were subsequently reduced to 0.5 g ion exchanger (1:2; m/m), and for Indion 234 and Indion 234S to 0.25 g (1:4; m/m) in 100 ml methylene blue solution (1%). These properties already showed the highest binding capacities for these ion exchangers.

3.3. Binding of methylene blue to ion exchange resins

In general the specific binding capacity of ion exchangers with sulphonic acid groups is smaller than with carboxyl groups because of their less favourable ratio of specific weight to number of binding sites. While monomer weights of acrylic acid (71 g/mol) and methacrylic acid (85 g/mol) are quite low per binding carboxyl group, styrene (183 g/mol) and divinylbenzene (203 g/mol) monomers are rather heavy. Together with a high affinity of methylene blue to carboxyl groups this implies that the specific binding capacity of sulphonic acid exchangers is smaller. This may explain why the binding capacity of Indion 254 and IRP 69 was limited compared to Indion 234, 234S, 294, and Amberlite IRP 88.

The binding of methylene blue chloride trihydrate to ion exchangers varied between 223 and 2160 mg/g ion exchange resin (Table 1) and the maximum loading capacity was reached within 15 min (Fig. 2). As expected, differences between the structurally identical ion exchangers Indion 234 and 234S, which showed the highest binding capacity, were small. This implies that the particle size did not influence the extent of binding significantly. No substantial difference was observed between Indion 294 and Amberlite IRP 88, which excludes relevant differences between both products even though produced by different manufacturers. The protonated Amberlite IRP 64 had a sevenfold lower

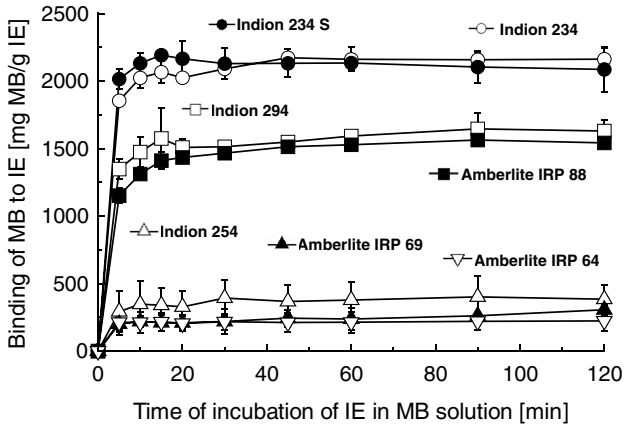

Fig. 2. Time course of binding of methylene blue chloride·3H_2O (MB) to seven ion exchange resins (IE) at room temperature.

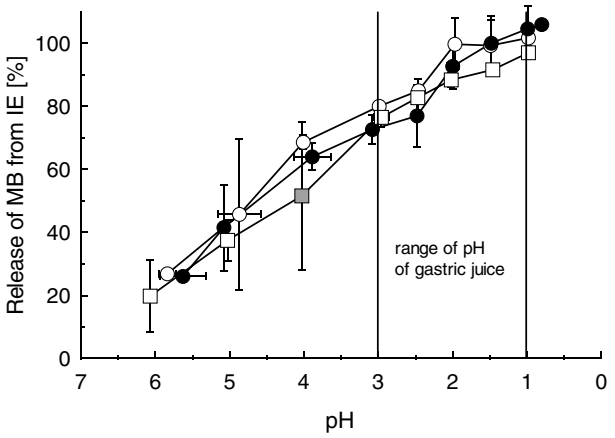

Fig. 3. Effect of pH modulation on the release of methylene blue (MB) from the ion exchange resins (IE) Indion 294 (-○-), Indion 234 (-●-), and Amberlite IRP 88 (-□-) in aqueous solution.

binding capacity compared to Amberlite IRP 88, the potassium salt of the same polymer suggesting that H^+ was less easily displaced by methylene blue than K^+ and that the release of bound methylene blue was strongly pH dependent.

The influence of elevated temperature (40 and 60 °C) and altered pH on binding capacity was tested because a further binding of methylene blue may expected when varying these parameters. However, elevated temperatures did not change the binding capacity of Indion 234, 234S, 294, and Amberlite IRP 88, but doubled the binding capacity of Indion 254 from 384 to 776 mg/g. Also Amberlite IRP 69 doubled the binding capacity at 40 °C and threefold at 60 °C from 306 mg/g (room temperature) to 648 mg/g and 987 mg/g, respectively (Table 2). Due to its generally low binding capacity IRP 64 was not tested. Mechanistically, this behaviour can be attributed to the occupation of inner binding sites reached by methylene blue at higher temperature. Cooling down to room temperature did not release methylene blue indicating that the binding capacity can safely be increased with higher temperatures of the selected ion exchangers. Alkalinisation did not increase binding capacity, while decreasing pH decreased the binding of Indion 234, 234S, 294, and Amberlite IRP 88 to one-third or even more by substitution of methylene blue by H^+ (Table 2). No substantial difference occurred with other ion exchange resins.

Table 2
Binding of methylene blue chloride trihydrate (MB) to ion exchangers (IE) at different temperatures and pH values

Ion exchanger (IE)	Maximum binding of MB to ion exchangers [mg MB/g IE]			
	40 °C/pH 7	60 °C/pH 7	RT/pH ~2	RT/pH >8
Indion 234	2089	2072	549	2067
Indion 234S	2099	1987	414	2149
Indion 254	420	776	272	269
Indion 294	1603	1657	769	1632
IRP 64	n.d.	n.d.	151	191
IRP 69	648	987	358	360
IRP 88	1565	1706	716	1546

n.d., not determined; RT, room temperature.

3.4. Release of methylene blue from ion exchange resins

The release of methylene blue was evaluated only for Indion 234, 294, and Amberlite IRP 88, because these ion exchangers had the highest binding capacities and therefore the highest potential of being considered as pharmaceutical carriers for methylene blue. Indion 234S was not studied because it only differs from Indion 234 in particle size. The release of methylene blue from ion exchangers should be studied at physiological conditions. However, the pH of gastric juice is highly variable. While in fasting healthy adults the pH is 2–3, the mean pH of fasting children aged 8–14 is about 1.5, never exceeds 3, and the mean gastric residence time is 1.1 h [15]. From these data most methylene blue should be released rapidly at pH values below 3. At pH 1.5 (gastric juice of children) complete releases of 100% from Indion 234 and 294 were observed (Table 1 and Fig. 3). Even at pH 3, more than 70% of bound methylene blue was released. Amberlite IRP 88 released 92% at pH 1.5 and 70% at pH 3. The release was independent of temperature (room temperature versus 37 °C). No differences occurred whether the pH was lowered slowly or the ion exchanger was transferred directly into a solution with pH 2 (equilibration time ~10 min.). The influence of food on the pH of gastric juice is not consistent, but depending on kind, volume, and buffer capacity. However, milk or Heilnahrung HN25 did not affect the pH-dependent release from Indion 234 (Fig. 4) and the release of methylene blue from Indion 294 was slightly lower with milk (~15%, Fig. 5).

4. Conclusion

Indion 234 and Indion 234S, polyacrylic cation exchange resins with carboxylic acid groups, had the best binding and release properties to fulfil the masking of tissue-colouring and bitterness. It binds more than twice its weight of methylene blue and release is complete at physiological pH values of the stomach. But also Amberlite IRP

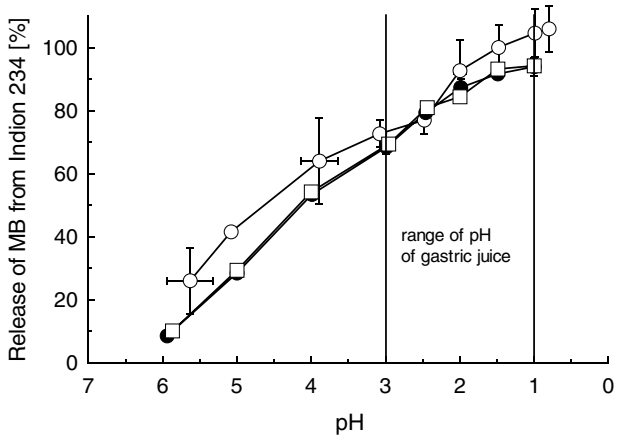

Fig. 4. Effect of pH modulation on the release of methylene blue (MB) from Indion 234 in water (-○-), milk (-●-), and baby food (Heilnahrung HN25; -□-).

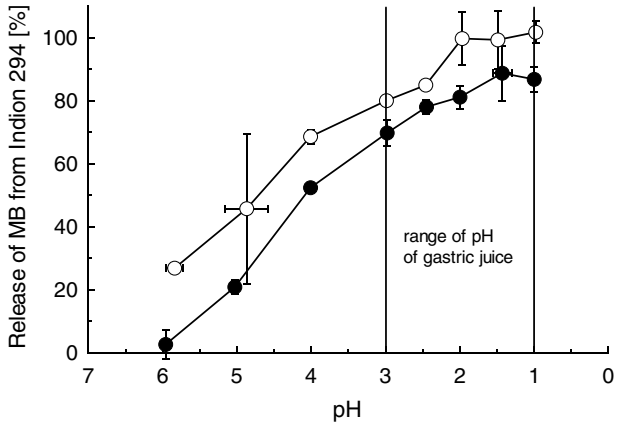

Fig. 5. Effect of pH modulation on the release of methylene blue (MB) from Indion 294 in water (-○-) and milk (-●-).

88 and Indion 294 are promising ion exchange resins with potential for further development as pharmaceutical carriers for methylene blue. The data indicate that for delivery of a dose of 100 mg methylene blue chloride trihydrate between 122 mg (Indion 234) and 147 mg (IRP 88) loaded ion exchanger must be administered.

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References

[1] P. Guttmann, P. Ehrlich, Ueber die Wirkung des Methylenblau bei Malaria, Berl. Klin. Wochenschr. 28 (1891) 953–956.

[2] R.H. Schirmer, B. Coulibaly, A. Stich, M. Scheiwein, H. Merkle, J. Eubel, K. Becker, H. Becher, O. Müller, T. Zich, W. Schiek, B. Kouyaté B, Methylene blue as an antimalarial agent – past and future, Redox Rep. 8 (2003) 272–275.

[3] P.M. Färber, L.D. Arscott, C.H. Williams, K. Becker, R.H. Schirmer, Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue, FEBS Lett. 422 (1998) 311–314.

[4] S. Meierjohann, R.D. Walter, S. Müller, Regulation of intracellular glutathione levels in erythrocytes infected with chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*, Biochem. J. 368 (2002) 761–768.

[5] H. Ginsburg, O. Famin, J. Zhang, M. Krugliak, Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action, Biochem. Pharmacol. 56 (1998) 1305–1313.

[6] M. Akoachere, K. Buchholz, E. Fischer, J. Burhenne, W.E. Haefeli, H. Schirmer, K. Becker, In vitro assessment of methylene blue on chloroquine sensitive and resistant *Plasmodium falciparum* strains reveals synergistic action with artemisinins, Antimicrob. Agents Chemother. 49 (2005) 4592–4597.

[7] J.L. Vennerstrom, M.T. Makler, C.K. Angerhofer, J.A. Williams, Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines, Antimicrob. Agents Chemother. 39 (1995) 2671–2677.

[8] C. Peter, D. Hongwan, A. Küpfer, B.H. Lauterburg, Pharmacokinetics and organ distribution of intravenous and oral methylene blue, Eur. J. Clin. Pharmacol. 56 (2000) 247–250.

[9] D.S. Chongtham, J. Phurailatpam, M.M. Singh, T.R. Singh, Methaemoglobinemia in nitrobenzene poisoning, J. Postgrad. Med. 43 (1997) 73–74.

[10] J. Rengelshausen, J. Burhenne, M. Fröhlich, Y. Tayrouz, S.K. Singh, K.D. Riedel, O. Müller, T. Hoppe-Tichy, W.E. Haefeli, G. Mikus, I. Walter-Sack, Pharmacokinetic interaction of chloroquine and methylene blue combination against malaria, Eur. J. Clin. Pharmacol. 60 (2004) 709–715.

[11] P.E. Meissner, G. Mandi, B. Coulibaly, S. Witte, T. Tapsoba, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, I. Walter-Sack, G. Mikus, J. Burhenne, K.D. Riedel, R.H. Schirmer, B. Kouyaté, O. Müller, Methylene blue for malaria in Africa: results from a dose-finding study in combination with chloroquine, Malar. J. 5 (2006) 84.

[12] G. Mandi, S. Witte, P. Meissner, B. Coulibaly, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, M. Sanon, K. Wüst, I. Walter-Sack, G. Mikus, J. Burhenne, K.D. Riedel, H. Schirmer, B. Kouyaté, O. Müller, Safety of the combination of chloroquine and methylene blue in healthy adult men with G6PD deficiency from rural Burkina Faso, Trop. Med. Int. Health 10 (2005) 32–38.

[13] P.E. Meissner, G. Mandi, S. Witte, B. Coulibaly, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, M. Sanon, T. Tapsoba, I. Walter-Sack, G. Mikus, J. Burhenne, K.D. Riedel, H. Schirmer, B. Kouyaté, O. Müller, Safety of the methylene blue plus chloroquine combination in the treatment of uncomplicated falciparum malaria in young children of Burkina Faso, Malar. J. 4 (2005) 45.

[14] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, May 2001. Available from: <http://www.fda.gov/cder/guidance/4252fnl.htm> (accessed September 26, 2007).

[15] J. Fallingborg, L.A. Christensen, M. Ingeman-Nielsen, B.A. Jacobsen, K. Abildgaard, H.H. Rasmussen, S.N. Rasmussen, Measurement of gastrointestinal pH and regional transit times in normal children, J. Pediatr. Gastroenterol. Nutr. 11 (1990) 211–214.