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Analysis of methylene blue in human urine by capillary electrophoresis

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Abstract

A capillary electrophoresis method for the determination of the dye methylene blue (tetramethylthionine, MB) in human urine depending on liquid/liquid-extraction and diode array detection has been developed, validated, and applied to samples of healthy individuals, who had been dosed with methylene blue within clinical studies. After extraction with dichloromethane and sodium hexanesulfonate, sample extracts were measured on an extended light path capillary. The dye was detected simultaneously at 292 and 592 nm using methylene violet 3 RAX as internal standard. The limit of quantification was 1.0 μg/ml. The accuracy of the method varied between −15.2 and +0.8% and the precision ranged from 2.0 to 12.0%. The method was linear at least within 1.0 and 60 μg/ml. In contrast to earlier indirect determinations no leuco methylene blue (LMB) was directly detected in urine, whereas in aqueous test solutions containing surplus amounts of ascorbic acid leuco methylene blue was well separated from MB in a single run.

Keywords: Capillary electrophoresis; Methylene blue; Leuco methylene blue; Human urine; Validation

1. Introduction

In view of the increasing drug resistance of malaria pathogens combination therapy with the antiplasmodial dye methylene blue (tetramethylthionine, MB) has recently been under clinical investigation [1], because it may be an effective and inexpensive alternative treatment. The pharmacokinetics of MB are not well characterized and quantification of MB in biological samples including urine was therefore deemed necessary in such a trial. In aqueous solutions, MB is in a redox equilibrium with its colourless reduced form leuco methylene blue (LMB) (Fig. 1). MB is almost exclusively eliminated by the kidneys resulting in high urine concentrations [2–5] and also the urinary elimination of LMB has been reported [2,3,5,6]. In these studies, LMB has only been determined in biological liquids by indirect measurements. The direct determination of both MB and LMB from human samples in a single analytical run remains an unsolved problem in view of the extraction of bound LMB [2], the ease of oxidation of LMB to MB [7,8], and possible demethylation products of MB [6]. Further on, LMB is not available as a standard substance and has only been obtained by diluting MB-solutions with reducing agents [4,7,9,10].

Numerous approaches have been reported to increase the selectivity and sensitivity of MB assays. The extraction of MB from urine was improved by adding sodium hexanesulfonate as ion-paring reagent [2–4] compared to sodium chloride and other inorganic salts [2]. Most analytical assays used HPLC with a wide range of columns including lipophilic RP18 columns [6,11–15], polar cyano columns [5,8], and an ion chromatography column [16] coupled to different optical [5,6,8] or mass spectroscopic detectors [14,15]. In spite of the superior separation efficiency of capillary electrophoresis (CE) and the ionic structure of MB only sporadic information of the electrophoretic behaviour of MB has been reported [17–22]. CE has not been used for routine determinations of MB in human samples thus far. Therefore, our aim was the development of a validated CE-method [23,24] for the quantitative determination of ionic MB in human urine in routine analysis with a commercial CE-instrument equipped with a diode array detector (DAD). The relatively high concentrations of MB reported in human urine in the course of clinical studies [1–5] should facilitate this task in view of the limited optical pathway and thus inferior detection limits of CE equipped with most optical absorption detectors

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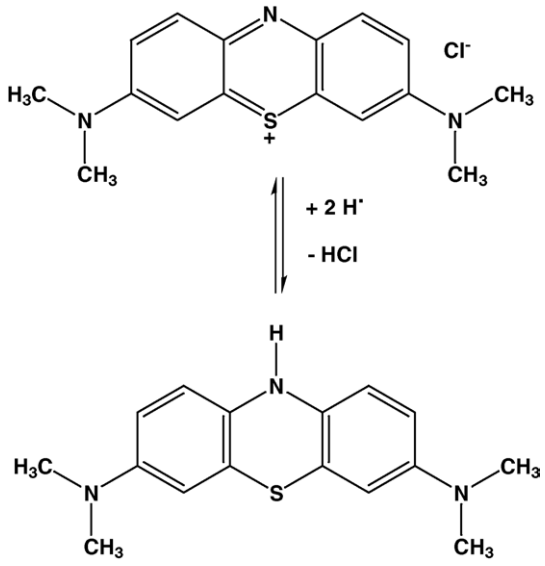

Fig. 1. Reduction of methylene blue (MB) to leuco methylene blue (LMB).

compared to HPLC. A secondary aim was the direct separation of MB from LMB at least in an artificial mixture in a single run.

2. Experimental

2.1. Chemicals

All chemicals were ordered from suppliers with offices in Germany.

2.1.1. All standard compounds

MB (purity >87%) was purchased from Calbiochem, Schwalbach, and the internal standard (I.S.) methylene violet 3 RAX (MV, purity ca. 90%) from Aldrich, Taufkirchen. Liquid chromatography-triple quadrupole investigations (LC/MS/MS) suggested an impurity of approximately 8% of azure B in MB. Azure B (no purity notified) was ordered from Fluka. LC/MS/MS suggested an impurity of less than 10% of MB in azure B.

2.1.2. Other chemicals

All of the reagents and solvents were of p.a. quality or higher with a purity of ≥99% including ascorbic acid, ammonium acetate, hexane-1-sulfonic acid sodium salt, sodium dihydrogenphosphate monohydrate, 50% ortho-phosphoric acid, and sodium dodecylsulfate (SDS). Optical grade (Uvasol, purity >99.9%) dichloromethane, methanol, and acetonitrile (Lichrosolv, purity >99.9%) were ordered from Merck, Darmstadt. High purity water (conductivity <0.55 μS) was supplied by a TKA laboratory unit (Niederelbert, Germany). Borate buffer solution (50 mM, pH 9.3) and 33 mM H_3PO_4 were ordered from Agilent, Waldbronn. pH buffer solutions (pH 2.0 and 7.0) were purchased from Riedel de Haen, Hannover, and Merck, Darmstadt.

2.2. Preparation of solutions and separation buffers

Volumetric flasks made of glass were used. Care was taken that the standard solutions, quality control solutions (QC), and the solution of lower limit of quantification (LOQ) did not come into contact with the highly adsorptive glass seals and connectors [12].

2.2.1. Standard solutions

MB (24 mg) was dissolved in 10 ml high purity water. This stock solution was diluted with high purity water resulting in concentrations from 1200 to 37.5 μg/ml. The stock solution was also added to urine to achieve the highest calibration level. Twelve milligrams of the I.S. MV were dissolved in 10 ml high purity water, resulting in a concentration of 1200 μg/ml.

2.2.2. QC- and LOQ-solutions

MB (24 mg) was weighed independently and dissolved in 10 ml high purity water, resulting in a concentration of 2400 μg/ml. Aliquots of this solution were diluted to 1200, 200, and 40.0 μg/ml (LOQ).

2.2.3. Ion-pair solution for extraction of MB and MV from urine

Hexane-1-sulfonic acid sodium salt (2.50 g) was dissolved in high purity water in a 50 ml volumetric flask, resulting in a 5% solution (m/v).

2.2.4. Separation buffer for electrophoresis and for comparative micellar electrokinetic chromatography (MECC)

$NaH_2PO_4 \cdot H_2O$ (6.90 g) was dissolved in 500 ml high purity water resulting in a 100 mM NaH_2PO_4 concentration which was transferred to a 1000 ml volumetric cylinder. This solution was adjusted to pH 2.50 by addition of 100 mM phosphoric acid which was prepared by dilution of 50% phosphoric acid. Acetonitrile was added to this buffer to make up 25% (v/v) of the entire separation solution. For comparative MECC [18] 10 ml of borate buffer (50 mM) were adjusted to pH 8.63 by addition of 33 mM H_3PO_4. SDS and methanol were added to obtain a concentration of 6% SDS (m/v) and 3% methanol (v/v). All solutions were stored at 6 °C.

2.3. Urine samples

Blank urine samples were supplied from eight volunteers for calibrations, QC- and LOQ-samples. One hundred twenty-four urine samples from 39 individuals besides numerous urine quality control samples were analyzed after approval of the study by the Ethics Committee of the Medical Faculty of the University of Heidelberg, after written informed consent of all volunteers was obtained, and after validation of our CE-method. Urine samples were immediately frozen after reception and stored at −20 °C until analysis.

2.4. Calibration-, QC-, and LOQ-samples

Analytical method validation was performed in accordance to international guidelines [23,24]. Frozen and thawed as well as fresh blank urine samples (2 ml) of eight different individuals were used.

2.4.1. Calibration samples

For each calibration the following procedure was performed: one blank urine sample was used as a blank control for both MB and MV. Another blank urine sample was only spiked with 50 μl of the I.S.-solution resulting in a urine concentration of 30 μg/ml of MV. Additional blank urine samples were spiked with 50 μl I.S.-solution and with 50 μl of one of the standard solutions of MB resulting in urine concentrations of MB of 0.94, 1.88, 3.75, 7.50, 15.0, 30.0, and 60.0 μg/ml.

2.4.2. QC- and LOQ-samples

Each of the blank urine samples was spiked with 50 μl of the I.S.-solution and with 50 μl of one of the QC-solutions or of the LOQ-solution. The resulting concentrations of MB in urine were 1.0 μg/ml (LOQ), 5.0, 30.0, and 60.0 μg/ml (QC-levels). The urine concentration of MV was always 30 μg/ml.

2.5. Sample preparation

For sample preparation the methods of recently reported [4,5] clinical studies were adopted. For this purpose, urine samples of 2 ml were spiked with 50 μl I.S.-solution in disposable culture glass tubes (12 ml) with screw caps and septa covered with a layer of inert Teflon. The 0.5 ml of 5% hexane-1-sulfonic acid sodium salt in water (m/v) and 7.5 ml dichloromethane were added for the extraction. The liquid phases were thoroughly mixed by the test tube rotator for 20 min. After a settling time of about 90 min almost all of the lower dichloromethane phase was transferred to a disposable evaporation tube of borosilicate glass and evaporated to dryness at 40 °C under a stream of nitrogen. The dried extracts were dissolved in 425 μl high purity water in order to benefit from stacking effects [25] during the injection of the samples into the CE-capillary. Due to the very high adsorptivity of MB neither the urine samples nor the extracts were filtered.

2.6. Reduction of aqueous test solutions of MB with ascorbic acid

An aqueous solution of MB (1280 μg/ml) was reduced with a 256-fold excess of ascorbic acid [10]. This solution (pH 3.06) was stepwise diluted with high purity water until a pH value of 4.91 was reached.

2.7. Capillary electrophoresis

A Hewlett-Packard ^{3D}CE-Instrument, Waldbronn, Germany, equipped with a deuterium lamp, and a diode-array-detector ranging from 190 to 600 nm was used. The detector was set to 292 and 592 nm with a bandwidth of 10 nm at each wavelength. Both of the wavelengths were used throughout the validation process as well as for the analysis of all of the urine samples. Optimisation of the CE-parameters resulted in a 100 mM phosphate buffer (pH 2.5) with 25% (v/v) acetonitrile as additive and a voltage of 19 kV. Extended light path (bubble) capillaries (effective length 56 cm, I.D. 75 μm) were used resulting in a field strength of 295.6 V/cm. Capillaries were kept at 16 °C. Prior to each run the capillary was conditioned with 100 mM phosphoric acid for 0.8 min and with the separation buffer for 6 min. Before each run the buffer vials were automatically replenished to avoid depletion of the separation buffer. Aqueous samples were injected to utilise sample stacking [25]. Hydrodynamic injection was performed for 2 s at 50 mbar pressure difference resulting in an injection volume of about 13 nl [26]. The ratios of the migration times $t_{\mathrm{mig\,MB}}/t_{\mathrm{mig\,MV}}$ were calculated to reduce the tolerances of the migration data [27]. Aiming for shorter run times a smaller extended light path capillary (I.D. 50 μm, total length 48.5 cm, effective length 40.0 cm) was initially applied with the same separation buffer at a field strength of 515 V/cm. Further on a comparative MECC-run [18] was performed with an extended light path capillary (effective length 56 cm, I.D. 75 μm) at 20.5 kV resulting in electrical field strength of 317.8 V/cm. The buffer vials were manually exchanged during these experiments to avoid bubble formation in view of the rather high SDS concentration (208 mM) [18] and depletion of the buffer.

2.8. Mass spectrometry

The HPLC system consisted of a quarternary LC pump (TSP model P4000, Thermo Electron, Dreieich, Germany) with degasser, autosampler (Gilson Abimed XL 232), and column heater. This HPLC system was coupled to a Thermo Finnigan TSQ 7000 tandem mass spectrometer via an electrospray ionisation interface. Purity measurements for the standard compounds MB and azure B were performed using a Kromasil C18 column 100 A 3 μm, 70 mm × 2 mm I.D. with integrated guard column. Eluents were 0.1% (volume) aqueous acetic acid including 20 mM ammonium acetate (A) and acetonitrile (B). The gradient used started with 95% of A (1 min isocratic) increasing linearly eluent B to 95% within 20 min. The mass spectrometer was set in the positive single MS mode (total ion scan m/z 150–500). For structure elucidation in reduced and non-reduced MB solution direct flow injection and positive electrospray with tandem MS (MS/MS, daughter ion scan) was used. The transfer capillary was set to 300 °C. Argon was used for CID-experiments.

2.9. Further instruments

A Knick pH-Meter, type 765 Calimatic (Berlin, Germany) was used to adjust the phosphate buffer to pH 2.50. The instrument was calibrated with buffer solutions of pH 2.00 and 7.00. A Heidolph Reax 2 test tube rotator (Schwalbach, Germany) was applied for the extraction of urine. Dichloromethane extracts were evaporated under a stream of nitrogen (99.995%) in a Zymark Turbovap LV apparatus (Caliper, Rüsselsheim, Germany).

2.10. Statistical evaluation

Statistical tests were performed using Microsoft Excel, office version XP.

3. Results

3.1. Comparison of the separation efficiencies of CE and MECC

The MECC-experiments of Jing et al. [18] already could be repeated in a bubble capillary column of 56.5 cm effective length and 75 μm I.D. at a field strength of 310 V/cm. A separation efficiency of 144,000 theoretical plates was achieved for MB at a total run time >56 min. In spite of a relatively high sample concentration of 40.2 μg/ml of MB an increased baseline noise was observed in agreement with the results of the authors [18]. The use of a separation mixture of 75% (v/v) 100 mM phosphate buffer (pH 2.5) and 25% (v/v) acetonitrile in a capillary of the same size at a field strength of 294 V/cm decreased the migration times by more than 50%. A separation efficiency of 23,800 theoretical plates was achieved for MB, 74,600 for MV and a resolution, *R* of 7.0 between both (Fig. 2).

By applying the same test mixture to a smaller capillary (I.D. 50 μm, effective length of 40.0 cm) at an electrical field strength of 515 V/cm a decreased separation efficiency of 20,000 theoretical plates for MB, 52,500 for MV, and a resolution, *R* of 3.8 was obtained at about half of the migration time. However, the smaller capillaries were not robust enough for the analysis of unfiltered urine extracts and their handling was difficult.

3.2. Repeatability of migration times in runs of urine extracts

The relative standard deviations of the absolute migration times of MB (mean 16.885 min) were 7.1% and of the internal standard MV (mean 19.667 min) 8.1%. The standard deviation of the migration time of MB relative to MV (mean 0.859) was reduced to 1.9%, however. In all batches the R.S.D. of the relative migration times of MB are considerably lower than the R.S.D. of the absolute migration times.

3.3. Calibration in urine matrix

Table 1 gives an overview of seven calibrations, each performed at the minor UV-maximum of MB at 292 nm and at 592 nm, i.e. the lower falling edge of the absorption maximum at 665 nm.

3.4. QC-samples, LOQ-samples, signal-to-noise ratios, linear range, and recoveries

The results of the six LOQ urine samples of six different individuals with a nominal concentration of MB of 1.0 μg/ml are shown in Table 2. The within-batch results of the three validation batches with a total of 18 urine QC-samples of eight individuals and the batch-to-batch results are listed in Table 3. MB was

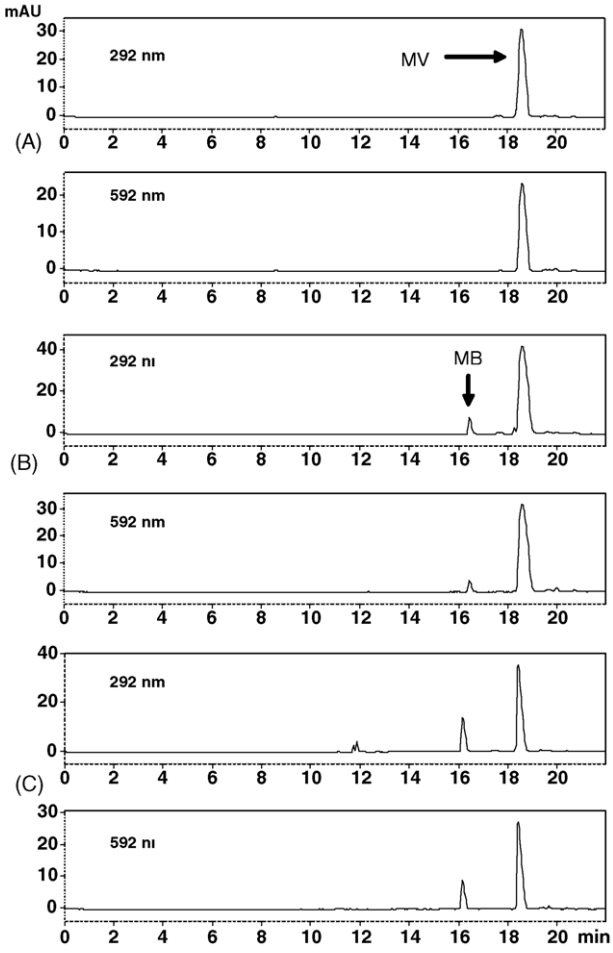

Fig. 2. Electropherograms of human urine extracts at detection wavelengths of 292 and 592 nm. Blank urine spiked: (A) with methylene violet 3 RAX (MV, I.S.) at 30 μg/ml; (B) with methylene blue (MB) at 1.0 μg/ml and with MV at 30 μg/ml. (C) Urine sample of a healthy individual exposed to oral MB. Conditions: fused silica capillary I.D., 75 μm; 64.5/56.0 cm total/effective length; bubble I.D., 200 μm; temperature, 16 °C; separation electrolyte, 75 vol% 100 mM phosphate buffer; pH 2.5; 25 vol% acetonitrile; voltage, 19 kV; hydrodynamic injection, 2 s/50 mbar.

detected with a S/N-ratio of 208 ± 8 at 292 nm and 51 ± 11 at 592 nm ($n = 6$) at the LOQ in urine. Considering the enrichment factor of 4.7 (2 ml urine: 0.425 ml extract) this corresponds to a calculated S/N of 44:1 at 292 nm and 11:1 at 592 nm in an aqueous test mixture. Liquid/liquid extraction using dichloromethane and the ion pair reagent hexane-1-sulfonic acid was used with respect to good results in recent clinical studies [4,5]. The recoveries varied between 42 and 66% at a urine concentration of 30 μg/ml and between 67 and 80% at a urine concentration of 60 μg/ml. Fig. 2 shows electropherograms of a urine blank sample, a LOQ run, and a urine sample from a clinical study at 292 and 592 nm. All samples were spiked with MV as I.S. at a urine concentration of 30 μg/ml.

3.5. Specificity and assignment of MB

In the course of the validation process blank urine samples of six volunteers were spiked at the LOQ level of 1.0 μg/ml and

Table 1
Overview of seven calibration regressions at 292 and 592 nm

Calibration lines $y = mx + b$ (y = peak area ratio (A_{MB}:A_{MV}); m = slope; b = intercept)								
Purpose	Calibration no.	292 nm			592 nm			n
		Slope	y-intercept	R^2	Slope	y-intercept	R^2	
Validation	1	0.0730	−0.0052	0.9998	0.0572	−0.0121	0.9995	8
Validation	2	0.0797	−0.0364	0.9975	0.0608	−0.0321	0.9975	8
Validation	3	0.0701	−0.0096	0.9994	0.0547	−0.0143	0.9997	8
Study K077	4	0.0743	−0.0035	1.0000	0.0573	−0.0082	1.0000	7
Study K102	5	0.0719	−0.0005	0.9998	0.0559	−0.0055	0.9998	8
Study K102	6	0.0721	−0.0328	0.9999	0.0586	−0.0474	0.9987	8
Study K102	7	0.0763	−0.0305	0.9998	0.0607	−0.0305	0.9997	8

Table 2
Accuracy and precision of MB at the LOQ-level (nominal concentration 1.00 μg/ml) at 292 and 592 nm (n = 6)

Parameter (nm)	292	592
Mean (μg/ml)	0.946	1.050
Median (μg/ml)	0.950	1.053
Accuracy (%)	−5.4	+5.0
Precision (CV, %)	3.9	2.8

analyzed. Both signals of 292 and 592 nm were used throughout the validation process as well as for the analysis of 124 urine samples of 39 volunteers of two clinical studies. The simultaneous observation of these two wavelengths substantially improved the assignment of peaks of this analytical method. This is demonstrated in Fig. 3 which shows an electropherogram of blank urine spiked with MB at the LOQ concentration. Fig. 3A represents the trace registered at 292 nm. Five peaks occur ahead of the huge peak of the I.S. Considering the R.S.D. of 2.3% of the relative migration times of this batch and a confidence interval of 2 S.D. still three of the five peaks in question of Fig. 3A remain in the corresponding time window (15.46–16.95 min). In contrast, the trace at 592 nm of the same sample only shows the peak of spiked MB with a migration time of 16.01 min (Fig. 3B). About 20% of the entire samples revealed small artefact peaks with similar migration times of MB at 292 nm well below 1 μg/ml assuming the same response at this wavelength. In contrast, hardly any artefact peak was detected at the trace of 592 nm and no artefact peak was registered for the I.S at either wavelength. No separation of MB (tetramethylthionine) from azure B (trimethylthionine) was achieved by employing our CE parameters.

3.6. Reduction of MB in test solutions with ascorbic acid

One milliliter of 100 mM ascorbic acid was added to 1 ml of a 0.39 mM solution of MB in water [10]. Within 5 min after addition of this 256-fold excess of ascorbic acid the dark blue colour of the MB solution faded to an almost colourless solution of a pH of 3.0. The electropherogram of this solution (Fig. 4A) revealed

Table 3
Within-batch results (n = 6) of MB-validation at QC-levels and batch-to-batch results (n = 7)

Batch		Nominal urine concentration					
		5.0 μg/ml		30.0 μg/ml		60.0 μg/ml	
		292 nm	592 nm	292 nm	592 nm	292 nm	592 nm
Within-batch results							
1	Mean (μg/ml)	4.51	4.48	27.6	27.4	54.8	55.6
	Median (μg/ml)	4.49	4.44	28.1	27.8	54.7	55.5
	Accuracy (%)	−9.8	−10.4	−7.9	−8.8	−8.7	−7.4
	Precision (R.S.D.%)	6.1	5.1	5.9	6.1	2.0	3.8
2	Mean (μg/ml)	5.00	5.04	26.4	26.2	50.9	51.3
	Median (μg/ml)	5.10	5.15	26.2	26.0	50.5	51.1
	Accuracy (%)	0.0	0.8	−12.0	−12.7	−15.2	−14.5
	Precision (R.S.D.%)	6.0	6.6	6.9	6.8	4.0	4.4
3	Mean (μg/ml)	4.59	4.62	29.2	28.7	56.0	55.3
	Median (μg/ml)	4.55	4.59	28.8	28.5	57.1	56.1
	Accuracy (%)	−8.2	−7.6	−2.8	−4.2	−6.7	−7.8
	Precision (R.S.D.%)	2.7	2.6	12.0	10.9	7.2	5.7
Batch-to-batch result							
	Mean (μg/ml)	4.70	4.72	27.7	27.4	53.9	54.1
	Median (μg/ml)	4.61	4.64	28.0	27.6	54.7	55.5
	Accuracy (%)	−6.0	−5.6	−7.7	−8.7	−10.2	−9.9
	Precision (R.S.D.%)	6.8	7.1	9.3	8.8	6.3	5.8

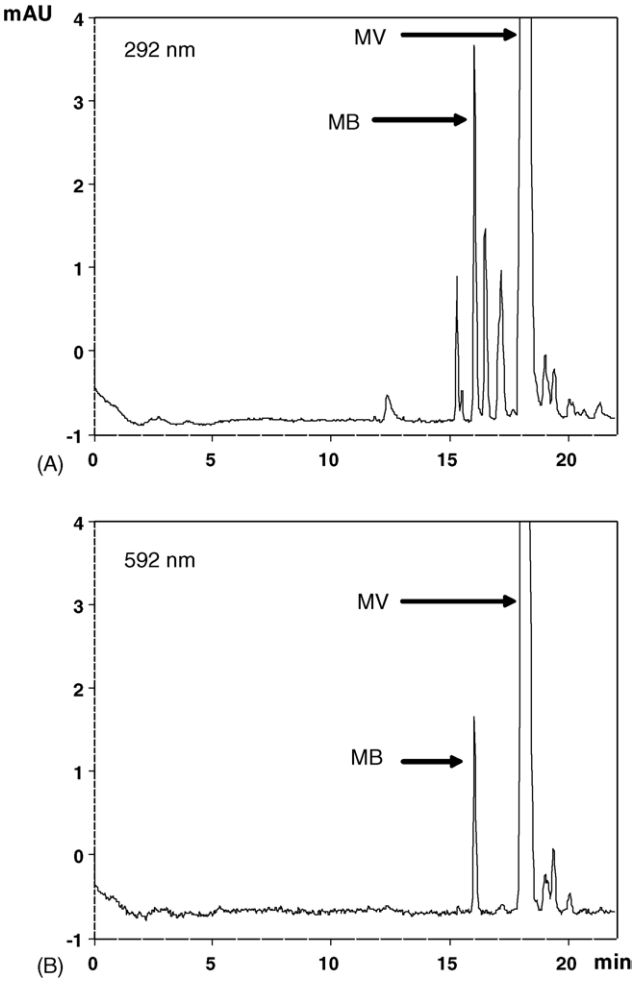

Fig. 3. Electropherogram of a blank urine sample spiked with methylene violet 3 RAX (MV, I.S., 30 μg/ml) and methylene blue (MB, 1 μg/ml) analysed at (A) 292 and (B) 592 nm. Conditions: fused silica capillary I.D., 75 μm; 64.5/56.0 cm total/effective length; bubble I.D., 200 μm; temperature, 16 °C; separation electrolyte, 75 vol% 100 mM phosphate buffer; pH 2.5; 25 vol% acetonitrile; voltage, 19 kV; hydrodynamic injection, 2 s/50 mbar.

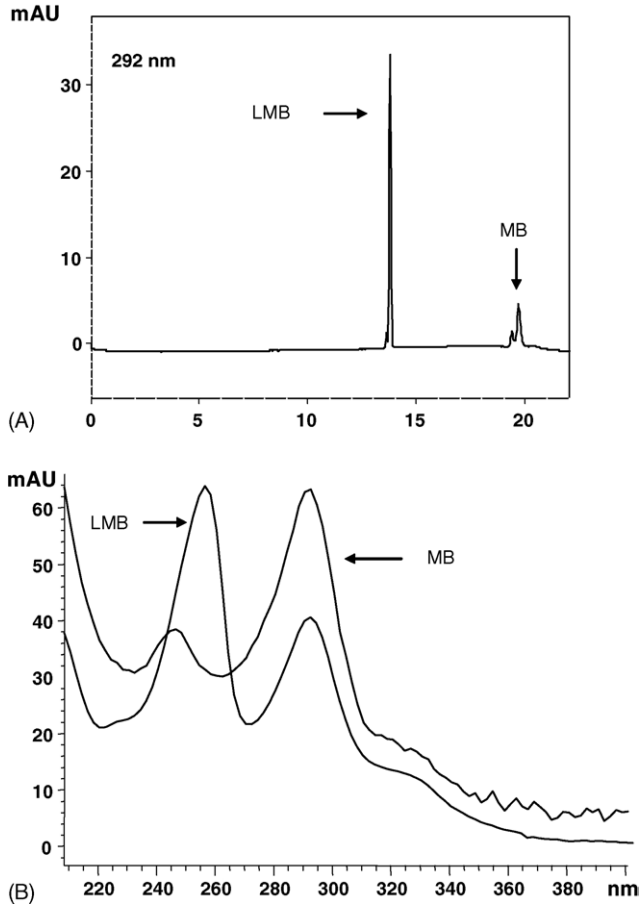

Fig. 4. Electropherogram at: (A) 292 nm and (B) UV spectra of methylene blue (MB) and leuco methylene blue (LMB) in ascorbic acid. Conditions: fused silica capillary I.D., 75 μm, 64.5/56.0 cm total/effective length; bubble I.D., 200 μm; temperature, 16 °C; separation electrolyte, 75 vol% 100 mM phosphate buffer; pH 2.5; 25 vol% acetonitrile; voltage, 19 kV; hydrodynamic injection, 2 s/50 mbar.

two major peaks at 13.77 and 19.69 min each of them preceded by a smaller peak. The peak at 19.69 min has the same migration time and the same UV/vis-spectrum with a minor maximum at 246.5 nm and a major maximum at 292.5 nm as MB. The dominant peak at 13.77 min shows a somewhat different UV-spectrum with a major maximum at 256.5 nm and minor maximum at 292.5 nm (Fig. 4B). The blank electropherograms of 100 and 10 mM ascorbic acid do not reveal any peak. Repeated reductions of MB starting with a 256-fold excess down to a 3.1-fold excess of ascorbic acid yielded very similar electropherograms and UV-spectra, although the latter reaction mixture still showed a bluish colour and a pH of 4.34 after 80 min.

4. Discussion

4.1. Separation buffer, capillary dimensions, and field strength

The separation mixture of 75% 100 mM phosphate buffer (pH 2.5) and 25% acetonitrile in combination with extended light capillaries of 56 cm length and 75 μm and a field strength of 294.6 V/cm proved to be a good optimisation for reasonable migration times of MB and MV, for high separation efficiencies for MB and MV, and for a satisfactory robustness for routine analysis in unfiltered urine extracts in view of the high adsorption of MB to various filter materials. While our CE parameters were independently established from earlier studies [19,20] separation voltage and buffer turned out to be similar. However, we chose acetonitrile as an additive to the acidic separation buffer because it has two advantages over alcohols in the analysis of MB. Firstly, acetonitrile does not have reducing properties, thus preventing the possible reduction of MB and MV during CE-runs. Secondly, acetonitrile does not react with acids to esters which may slowly change the composition of the separation buffer and thus the migration times in the course of long sample sequences [19,20]. Although MECC with relatively high amounts of SDS in the separation buffer exhibited a superior separation efficiency we doubt its usefulness for the routine analysis of low concentrations because of greatly increased baseline noise, substantially prolonged migration times, and the necessity of manual replenishment of the buffer. Smaller extended light path capillaries (effective length 40 cm, I.D. 50 μm) were

not robust enough for routine analysis of MB in urine in spite of the option of using higher field strengths which generally result in shorter run times.

4.2. LOQ, detection limit, and recovery

The LOQ of 1 μg/ml in urine was sufficient in several clinical studies [1,3–5], because humans excrete more than 50% of administered MB in urine [3]. In comparison to our results Hu et al. [20] reported a lower detection limit (10 ng/ml; S/N = 2) for MB by applying an indirect photothermal interference detector with an estimated 5–10-fold lower detection limit compared to the DAD of our CE instrument. On the other hand, our method is 10 times more sensitive than the assay of Hamai and Sato [21] which had an inferior detection limit of approximately 10 μg/ml, although the authors detected MB at its most intense absorption maximum at 660 nm. A LOQ of 10 μg/ml would have missed detection of MB in 17% of all our urine samples.

The recoveries varied between 42 and 80% partly depending on concentration. This is in accordance with recent studies [4,5] and requires the use of an appropriate internal standard. Indeed, the rather good accuracy data (Tables 2 and 3) confirms that methylene violet 3 RAX as an internal standard with very similar properties compared to methylene blue very well reflects the loss of analyte during sample processing. Table 3 additionally reveals that the accuracy tends to worsen with increasing concentrations of MB within the accepted limits of the validation process. This is in agreement with earlier findings [4] and can be explained by adsorption of MB especially on plastic surfaces, whereas MB solutions are stable in polyethylene bottles at

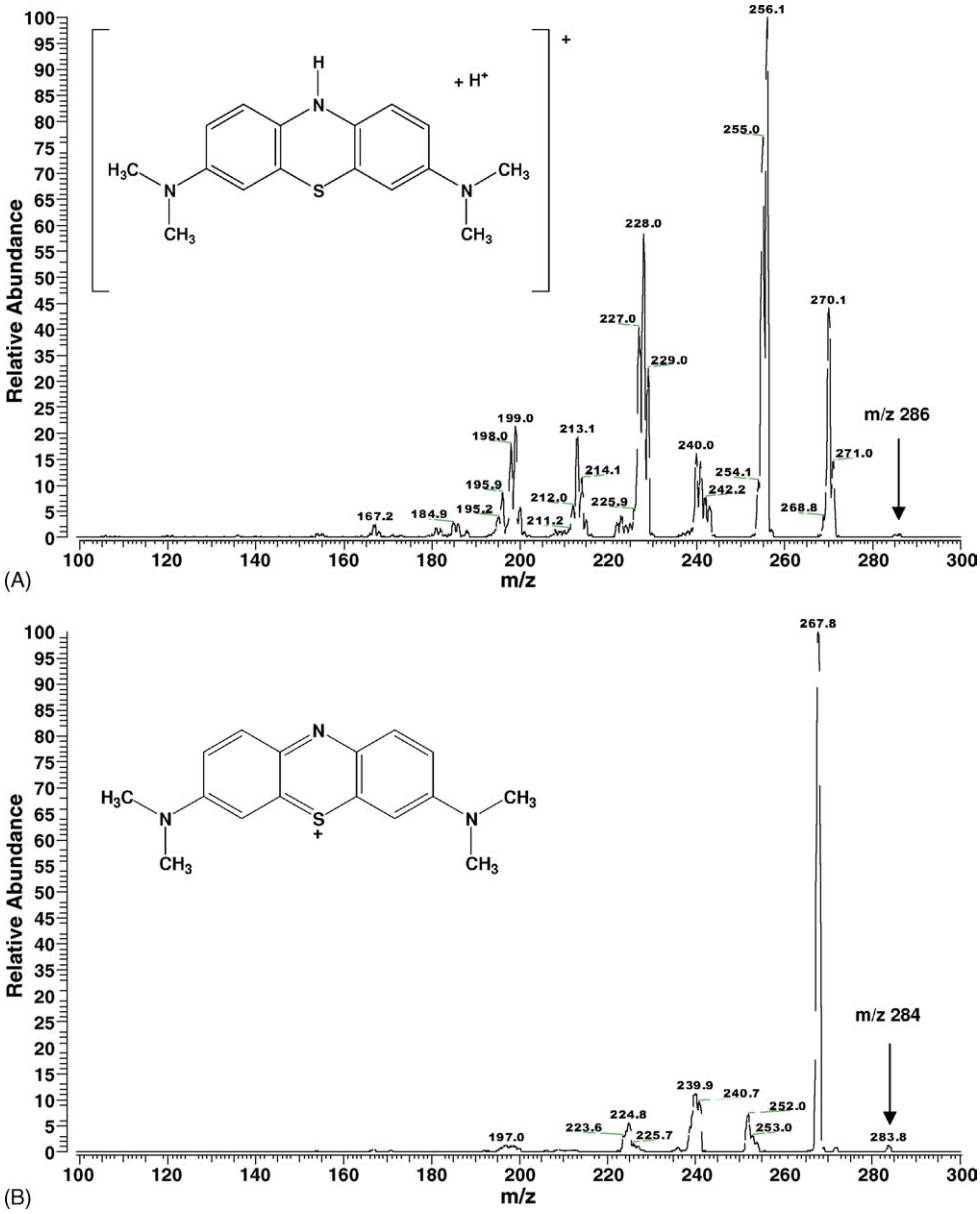

Fig. 5. ESI-MS/MS spectra of: (A) leuco methylene blue and (B) methylene blue in the daughter ion scan mode. Collision induced fragmentation was achieved by argon (2.0 mT) and 45 V offset voltage.

refrigerator temperatures [16]. Another reason for this loss may be the limited water solubility of MB at increased concentrations and low temperatures.

4.3. Specificity and assignment of MB in urine extracts

The findings in large series of analyzed urine samples indicate that MB can more easily be assigned by tracing the synchronized peaks for MB at both wavelengths of 292 and 592 nm at low concentrations rather than using relative migration times [27]. The analysis of our samples revealed that the LOQ of MB at 292 nm is mainly caused by chemical interferences, whereas the LOQ at 592 nm is caused by electronic noise. Thus, the less intense signal of 592 nm is much more specific for MB than the more intense signal of 292 nm. Since our assay was validated at 292 nm and at 592 nm, the ratio of peak areas for both wavelengths may serve as an additional criterion for the presence of MB in urine samples.

4.4. Reduction of MB in aqueous ascorbic acid solutions

The appearance of an additional, dominating, and well separated peak from MB upon its reduction with an excess of ascorbic acid (Fig. 4A) with a UV-spectrum exhibiting two absorption maxima at 256.5 and 292.5 nm is a strong indication for the occurrence of LMB (Fig. 4B). Measuring at 592 nm results in a similar electropherogram. A small peak for MB is still remaining, since the sample handling and processing was not performed under oxygen-free conditions. Partial oxidation of leuco methylene blue back to MB can be expected to occur because of this reversible process. The separation of LMB as an additional dominating peak might have been facilitated by the transport of the analytes to the reducing cathode of our CE in contrast to previous HPLC-experiments [8]. The reduction of MB by ascorbic acid resulting in LMB was additionally confirmed by tandem mass spectrometry and electrospray ionisation (ESI-MS/MS) using flow injection. The corresponding mass spectra (daughter ion scan) of MB ($m/z = 284$ $[M]^+$) and LMB ($m/z = 286$ $[M + H]^+$) are shown in Fig. 5.

Several limitations require mentioning. Although we were able to separate LMB from MB in an artificial test mixture we could not determine both analytes in a single run from urine samples probably due to the fast oxidation of LMB to MB [7–9] and lacking LMB standard substance. In this context, we observed that colourless acidic solutions of LMB turned blue upon the addition to surplus urine after a few minutes which is in agreement with earlier findings [8,9].

5. Conclusions

The validation according to accepted standards [23,24] and the subsequent determination of MB in urine extracts of humans exposed to therapeutic doses of MB was demonstrated using a commercial CE instrument equipped with a DAD covering the wavelength range from 190 to 600 nm. Moreover, in contrast to all other assays reported so far the presented method separates LMB from MB in ascorbic acid solutions.

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