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Journal of Chromatography B, 863 (2008) 273–282
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of cationic anti-malaria agent methylene blue in different human biological matrices using cation exchange chromatography coupled to tandem mass spectrometry

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Received 19 August 2007; accepted 16 January 2008
Available online 26 January 2008

Abstract

Selective and sensitive methods for the determination of the cationic dye and anti-malarial methylene blue in human liquid whole blood, dried whole blood (paper spot), and plasma depending on protein precipitation and cation exchange chromatography coupled to electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) have been developed, validated according to FDA standards, and applied to samples of healthy individuals and malaria patients within clinical studies. Acidic protein precipitation with acetonitrile and trifluoroacetic acid was used for liquid whole blood and plasma. For the extraction of methylene blue from paper spots aqueous acetonitrile was used. Sample extracts were chromatographed on a mixed mode column (cation exchange/reversed phase, Uptisphere MM1) using an aqueous ammonium acetate/acetonitrile gradient. Methylene blue was quantified with MS/MS in the selected reaction monitoring mode using ESI and methylene violet 3RAX as internal standard. Depending on the sample volume (whole blood and plasma 250 μL, and 100 μL on paper spots) the method was linear at least within 75 and 10,000 ng/mL and the limit of quantification in all matrices was 75 ng/mL. Batch-to-batch accuracies of the whole blood, plasma, and paper spot methods varied between −4.5 and +6.6%, −3.7 and +7.5%, and −5.8 and +11.1%, respectively, with corresponding precision ranging from 3.8 to 11.8% CV. After a single oral dose (500 mg) methylene blue concentrations were detectable for 72 h in plasma. The methods were applied within clinical studies to samples from healthy individuals and malaria patients from Burkina Faso.

Keywords: Methylene blue; Cation exchange chromatography; Tandem mass spectrometry; Whole blood; Plasma; Paper spots

1. Introduction

The use of the thiazine dye methylene blue (MB) for the treatment of malaria has been described over 100 years ago [1,2]. The mechanism of its anti-malarial activity is not fully understood, but MB has been shown to inhibit the glutathione reductase of *Plasmodium falciparum* more effectively than human glutathione reductase [3]. Inhibition of glutathione reductase leads to a decrease in intracellular levels of reduced glutathione, which is essential for the degradation of haematin [4,5]. Reduced degradation might result in an accumulation of haematin, which leads to poisoning of the parasites. In vitro experiments have confirmed the anti-malarial potency of MB alone and in combination with other anti-malarials [6]. Compared with other thiazine dyes MB yielded the highest activity and selectivity against different isolates of *P. falciparum* with IC50 values of approximately 4 nmol/L [7]. Whole blood concentrations of up to 100 nmol/L (=28.4 ng/mL) were reached in healthy individuals after oral administration of 100 mg MB [8], which is a dose usually applied for the oral treatment of methaemoglobinaemia [9]. On the basis of the in vitro results this dosage is expected to yield anti-malarial effectiveness. For dose finding, the evaluation of pharmacokinetics, and the safety of MB co-administered to a typical chloroquine regimen clinical stud-

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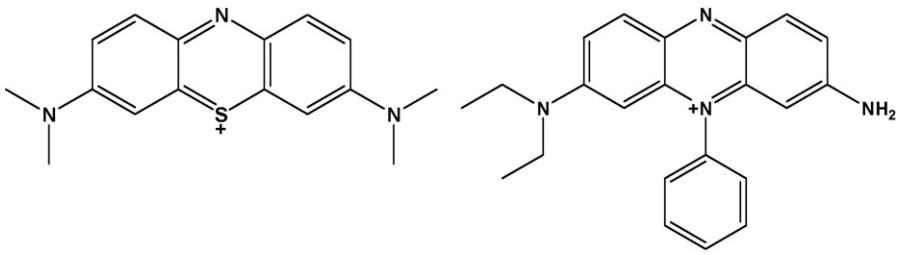
doi:10.1016/j.jchromb.2008.01.028



methylene blue
(analyte)
CAS 61-73-4
$[C_{16}H_{18}N_3S]^+$
Exact mass: 284.12
Mol. Wt.: 284.40

methylene violet 3RAX
(internal standard)
CAS 4569-86-2
$[C_{22}H_{23}N_4]^+$
Exact mass: 343.19
Mol. Wt.: 343.33

Fig. 1. Chemical structures and characteristics of the cations methylene blue (analyte) and methylene violet 3RAX (internal standard).

ies were performed [10–13]. Within these and further studies analytical quantifications of MB were necessary for pharmacokinetic assessment and documentation of compliance. Because the assay was also to be used in blood samples of children, high sensitivity and selectivity in combination with fast quantification in low sample volumes of whole blood and plasma were required. Another demand was MB quantification from paper spots, which were prepared for safe and easy transfer of originally infectious blood samples from Burkina Faso (Africa) to the laboratory in Heidelberg (Germany). For this purpose whole blood (100 μL) was spotted on filter paper and dried, which is a usual procedure [14–16]. The analytical methods had to ensure quantitative extraction and selective determination separated from potential artefacts caused by extraction from paper and biological matrix. Previous studies in which MB had to be analysed in biological matrices used high-performance liquid chromatography (HPLC) with UV-detection at high wavelength (>600 nm) and liquid/liquid extraction with chlorinated organic solvent [8,17]. Quantification in urine has been performed using capillary electrophoresis with UV-detection and liquid/liquid extraction with chlorinated organic solvent [18]. To our knowledge liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) has never been applied to MB quantification in biological matrices apart from our previous studies [6,10–13]. Chromatography of ionic analytes in general including MB (Fig. 1) should usually be performed using ion pair chromatography or ion exchange chromatography. Both do not really fit the demands of ESI-MS/MS which excludes non-volatile ingredients from the liquid phase. On the other hand reversed phase chromatography of polar ionic analytes without ion pair reagent will lead to dead volume elution. Therefore, in this paper we describe the development, validation [19,20], and application of ion exchange chromatography using a mixed mode phase onto cationic methylene blue. Although highly concentrated volatile buffers in gradient mode and extracts from biological matrices were applied to the ESI-MS/MS system no contamination of the ion source occurred. Quantification of MB is also challenging because of the dye's adsorptive properties during the sample preparation process which causes binding to different surfaces (e.g. glass, plastic, and endogenous substances like proteins). In previous studies [8,10] very low concentrations of MB in plasma were determined. We hypothesised that a large proportion of available MB will build up bound residues which will not be released with common liquid/liquid extraction procedures. In this paper we describe extraction procedures which led to more concentrated extracts by liberating more MB from the biological matrices. This was achieved by addition of acids or cationic reagents to displace MB from its counter ion. Hence it can be assumed that the use of former methods resulted mainly in the determination of the free fraction of MB in the respective matrix.

2. Clinical studies, participants, materials, and methods

2.1. Clinical studies and participants

The study protocols were approved by the Ethics Committee of the Medical Faculty of Heidelberg. The studies were conducted by the Departments of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology, and of Tropical Hygiene and Public Health in accordance with good clinical practice guidelines, the Declaration of Helsinki, and local legal requirements. For the determination of pharmacokinetic parameters healthy individuals received MB orally (500 mg) and intravenously (50 mg). The pharmacokinetic profiles of MB in plasma of one individual after oral and intravenous administration are shown in this paper; the pharmacokinetic results will be published elsewhere.

Within a clinical study in Burkina Faso, 20 paediatric patients received MB 20 mg/kg body weight MB orally for malaria therapy. Whole blood samples (100 μL) 1 h after administration were spotted onto filter paper, dried, stored darkly at room temperature, and transferred to Heidelberg for analysis.

2.2. Materials

The aqueous solution of MB containing 25 mg/mL was supplied by Mayrhofer Pharmazeutika GmbH (Linz, Austria) under contract of DSM Fine Chemicals Austria (Linz,