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Sensitive quantification of diphemanil methyl sulphate in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A simple detection system with a high-performance liquid chromatography (HPLC) with positive ionisation-tandem mass spectrometry (ESI-MS/MS) for determining diphemanil methylsulphate (DMS) levels in human plasma using 4-diphemanylmethylene,1-methylpiperidine as an internal standard (I.S.), is proposed. The acquisition was performed with the multiple reactional monitoring (MRM) mode, by monitoring the transitions: *m*/*z* 278 > 262 for DMS and *m*/*z* 263 > 247 for the I.S. The method involved a simple single-step deproteinisation with acetonitrile. The analyte was chromatographed on a Zorbax® C18 reversed-phase chromatographic column by isocratic elution with 10^{-3} M ammonium acetate and 10^{-3} M hexafluorobutyric acid, adjusted to pH 7.0 with ammoniac/acetonitrile (40/60, v/v). The results were linear over the studied range (0.5–50.0 ng mL^{-1}) and the total analysis time for each run was 10 min. The mean extraction apparent recoveries expressed at the 95% intervals of confidence were 94–104% for DMS and 92–106% for the I.S. The intra- and inter-assay precisions were 4.6–8.4% and 2.9–10.6%, respectively. The limit of quantification was 0.15 ng mL^{-1}. The devised assay was successfully applied to the residual concentrations monitoring in infant.
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1. Introduction

Diphemanil methylsulphate is a synthetic quaternary ammonium compound with antimuscarinic properties (4-(diphenylmethylene)-1,1-dimethylpiperidinium methylsulphate) (Fig. 1). It was used in the past in adults in the treatment of peptic ulcer, gastric hyperacidity and hypermotility, in gastritis and pylorospasm and in the treatment of hyperhidrosis.

In France, diphemanil methylsulphate constitutes a medicinal treatment in malaises with intense and repeated episodes of bradycardia related to vagal hyperexcitability in premature new born. The vagal bradycardia has been suggested to play a role in the pathogenesis of the sudden infant death syndrome [1,2]. In spite of severe adverse effects to the heart, this drug must be used with clinical monitoring and in the absence of a therapeutic alternative treatment.

The precautions consist of several medical recommendations before use, published in a release of the French regulatory authority (AFSSAPS) [3], such as electrocardiogram measurement. In certain cases, clinicians ask for measurements of diphemanil residual concentrations in plasma. However, very few analytical methods are available to quantify the studied analyte in biological fluids with sufficient sensitivity and specificity, when residual plasma concentrations in infants are not expected up to 10 μg L^{-1} [4,5].

In order to determine kinetic parameters in infants after oral drug administration, Vidal et al. [4] quantified diphemanil by gas–liquid chromatography method using an alkali flame ionisation detector after extraction under iodide ion pair form [4,5]. A limit of detection at 2 μg L^{-1} gives some information about the performance of the method but remains insufficient for the accurate residual concentration monitoring. In fact, a number of plasma samples to be analysed have been shown to present lower concentrations of DMS than this limit. Houri et al. have described an analytical procedure using a liquid chromatography for the stability monitoring of the active substance in an oral solid pharmaceutical form [6]. However, our preliminary studies showed that this chromatographic assay could not easily intend to the analyses of DMS in plasma because of its poor selectivity.

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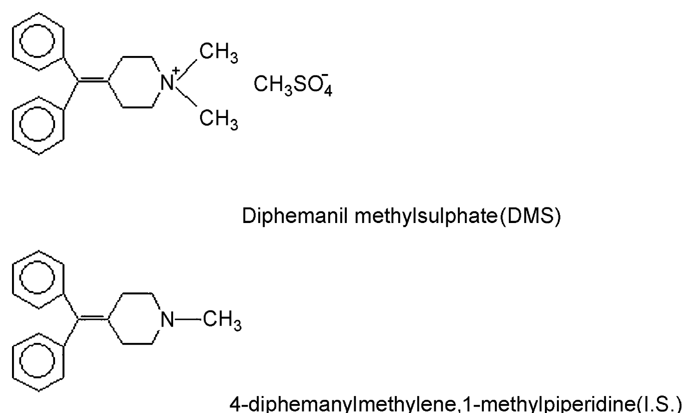

Fig. 1. Chemical structures of DMS and I.S.

Consequently, to routinely quantify DMS in plasma samples issued from taking of blood in infant the volumes of which could not exceed 250 μL, a rapid, specific and sensitive method is proposed here. The implemented method was a high-performance liquid chromatography coupled to the mass spectrometry in tandem. The chromatography was performed after deproteinisation of the plasma samples.

2. Experimental

2.1. Analytical standards and reagents

Diphemanil methylsulfate (purity ≥ 99%) and 4-diphemanylmethylene,1-methylpiperidine, used as I.S., were obtained from Schering–Plough Laboratories (Levallois–Perret, France). Acetonitrile and hexafluorobutyric acid were of HPLC grade (Riedel-de Haën and Sigma–Aldrich, St. Quentin Fallavier, France), ammonium acetate and ammoniac were of analytical grade (Merck, Darmstadt, Germany).

Deonised water from an ultrapure water system Milli-Q (Waters, Molsheim, France) was used for the preparation and the dilutions of all reagents, sample and calibration solutions.

2.2. Chromatographic and detection systems

2.2.1. Chromatography

An HPLC system (Thermofinnigan, Les Ulis, france) consisting of a flow control valve, a vacuum degasser, a pump and an autosampler was used to deliver a mobile phase (solvent A: ammonium acetate 10 mM and hexafluorobutyric acid 10 mM adjusted to pH 7.0 with ammoniac, solvent B: acetonitrile, in the ratio 40/60, v/v) at a flow-rate of 1.0 mL min^{-1}. The chromatographic separations were achieved on a Zorbax C18 stable bond column (75 mm × 4.6 mm i.d., 5 μm) preceded with a guard colum packed with the same material (20 mm × 4.6 mm i.d., 5 μm). The detection system used for different tests and preliminary assays was a photo-diode array detector UV 6000 LP. The samples (50 μL) were injected onto the LC–MS–MS system through an autoinjector.

2.2.2. Mass spectrometry

The mass spectra were obtained using a Finnigan LCQ ion trap mass spectrometer (Thermifinnigan, Les Ulis, france) equipped with an electrospray ionisation (ESI) source operating in positive ionisation (PI) mode. The data system was the Xcalibur® software version 4.01 (Thermoquest, San Jose, U.S.A.).

The optimum PI ESI MS parameters were as follows: probe adjustment position: 3; spray voltage: 4.5 kV; sheath gas flow-rate: 20 arbitrary units; capillary voltage: 30.00 V; capillary temperature: 200 °C; tube lens offset: 5.00 V; lens voltage: −16.00 V; multipole offset: −2.75 V; multipole 2 offset: −6.00 V.

For the optimum MS/MS performance, the collision energy was set to be 1.5 eV peak-to-peak of resonance excitation RF voltage to produce a nearly 100% fragmentation of the M^+, *m/z* 278 of DMS and obtain the maximum intensity of a product ion at *m/z* 262.

2.3. Preparation of samples

2.3.1. Preparation of standard solutions and calibration samples

Stock solutions of DMS and the I.S. were prepared by dissolving accurately weighed quantities in deionised water to give a concentration of 1 mg L^{-1}. The stock solutions were used to spike the drug-free plasma to cover the calibration range: 0.5–50.0 μg L^{-1}.

During the routine analysis, each analytical run included a set of calibration standards, a set of quality controls (QC) in duplicate and a set of plasma to be analysed.

2.3.2. Deproteinisation of plasma samples

The calibration and the control solutions were obtained by adding to 250 μL of plasma, 100 μL of a stock solution of DMS and 20 μL of the stock solution of the I.S.

The samples to be analysed were prepared by adding 100 μL of water and 20 μL of the stock solution of the I.S.

Deproteinisation was achieved by adding 750 μL of acetonitrile to these aqueous solutions. The tubes were capped, vortexed for 1 min and then centrifuged for 10 min at 4000 rpm. The supernatant was dried under nitrogen at 50 °C. Two hundred microlitres of mobile phase was added to residues and transferred into a 250 μL micro insert. Fifty microlitres was injected onto the LC–MS^2 system.

2.3.3. Apparent recovery

Various amounts of DMS of the concentration range investigated were dissolved in 250 μL of human drug-free plasma by adding 100 μL of appropriate working solutions. These samples were treated as described above. The deproteinisation supernatant obtained was dissolved in 200 μL of mobile phase and chromatographed.

A second series of samples was prepared simultaneously by deproteinisation of 250 μL aliquots of human drug-free plasma. DMS and the I.S. were then added to the deproteinisation supernatant at the concentration noted above.

The second series corresponds to the reference calibration range.

The analytical apparent recovery [7] for DMS was calculated by comparing the slope related to the calibration curve obtained from the first described series to the slope corresponding to the reference calibration curve. The calibration curves were determined with the signals of DMS without weighting by those of the I.S. The signals of the I.S. obtained for each concentration of DMS in plasma was compared to its signals from the reference series.

2.3.4. Quantification

Validation runs were conducted on 3 separate days. Each validation run consisted of a set calibration standards at 7 concentrations over the concentration range (0.5–50.0 μg L^{-1}, each in triplicate) and QC samples at three concentrations (5.0, 20.0 and 40.0 μg L^{-1}, $n = 6$ at each concentration). The results from QC samples were used to evaluate the accuracy and precision of the developed method.

The drug concentrations in plasma samples were determined by back-calculation of the observed peak area ratios of the drug and the I.S. from the best fit calibration curve.

The linearity of each calibration curve was determined by plotting the peak area ratio of the drug to the I.S. *versus* the nominal concentration of the analyte.

The validation of the method was performed by following the recommendations of the Pharmaceutical Sciences and Technical French Society (SFSTP) [8–10] and the International Conference of Harmonisation (ICH) [11,12].

3. Results and discussion

3.1. Performance of the HPLC system

Several approaches also including that described by Houri et al. [6] were simultaneously run to allow a rapid screening of the HPLC methods.

DMS is an ammonium quaternary compound with hydrophilic properties. The capability for DMS to be involved in hydrogenous bond formation with the silica gel hydroxyl groups was first exploited in an adsorption-phase chromatography. However, resolution factor between DMS and the I.S. could not exceed 1.5 whatever were the conditions used. Analyses performed by a normal-phase chromatography as was proposed by Houri et al. [6] did not allow separation of the drug from residual plasma compounds remained after the deproteinisation step and from the I.S.

Owing to the presence of a quaternary ammonium function conferring an apparent positive charge, DMS was analysed by ion-exchange chromatography. However, in these conditions it was not separated from the I.S. at pH $\leq$ 4.0 although different capacities of the exchange system were tested. At neutral pH, the I.S. was eluted in the dead volume.

The log p value of the active substance (2.57) might indicate that its interaction on a reversed-phase stationary phase would be meaningful and this was confirmed by HPLC analysis on an octadecylsilyl silica gel (C18) column without ion pair

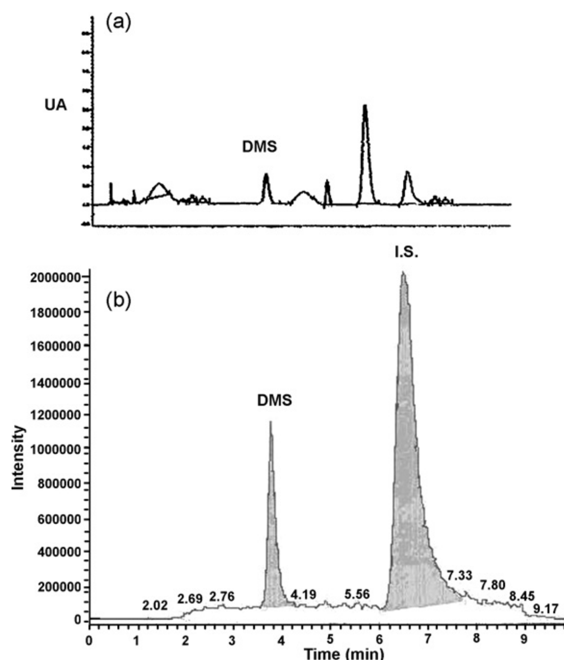

Fig. 2. (a) Analysis of plasma spiked with 1.0 μg mL^{-1} of DMS by HPLC-UV (254 nm), (b) analysis of plasma spiked with 1.0 μg mL^{-1} of DMS by HPLC–MS/MS.

reagents. However, on octylsilyl (C8) or octadecylsilyl (C18) silica gel column, DMS was characterised by asymmetric peaks with important signal trail. Measurements involving ion pair liquid chromatography gave fair results in a pH range $\geq$7.0, as peaks were symmetric and DMS and the I.S. were well resolved. With the use of octanesulfonate, the capacity factor of the drug was increased as a function of the concentration of the ion pair reagent in the range of 10^{-3}–10^{-1} mol L^{-1} whatever the pH of the mobile phase was. At pH < 5.0, the I.S. was not separated from DMS owing to their very close structures, but since it was mainly under the molecular form, the difference based on the apparent charge became meaningful.

As the hyphenation of HPLC to mass spectrometry required the use of a mobile phase devoid of non-volatile salts, octanesulfonate was replaced by hexafluorobutyric acid at a similar molar concentration.

In both cases, contrary to expected retention mechanisms of DMS, the interaction phenomena with the stationary phase were likely and mainly due to a dynamic ion-exchange, insofar as very slight modifications of capacity factor was noted with the ratios acetonitrile/buffer, whereas significant changes were observed in the presence of ammonium acetate. Finally, under the chromatographic conditions described above (Section 2.2.1), DMS and the I.S. were well resolved and interferences with endogenous compounds were not observed as shown in Fig. 2a and b despite a short analysis time inferior to 10 min. This was verified on five different batches of plasma.

To the best of our knowledge, no data on *in vivo* or *in vitro* metabolism of diphemanil are actually available. However, studies on pharmacokinetics of DMS in healthy subjects suggest bioavailability assuming that the drug is poorly metabolized [3,4].

The choice of such I.S. led to ensure that it is different from potential and unknown metabolites of the drug. Preliminary assays were undertaken on plasma samples of patients treated with DMS. The samples taken from plasma of children treated by oral administration of 10 mg per day of DMS were analysed using the above described procedure however with the lack of addition of the I.S. At the sampling time corresponding to the plasma residual concentration of the drug, 4-diphemanylmethylene,1-methylpiperidine was not detected.

3.2. Optimisation of MS and MS/MS conditions

Owing to the presence of a quaternary ammonium and a tertiary amine functional group on DMS and the I.S., respectively, the positive ionisation mode was chosen.

The molecular ion M^+, *m/z* 278 and the protonated molecular ion $[M+H]^+$, *m/z* 264 were obtained as the base ions for DMS and the I.S., respectively. No significant solvent adduct ions or fragment ions were observed in the full scan spectra of both the compounds.

In the product spectra of M^+ for DMS and $[M+H]^+$ for the I.S., along with the raising of the collision induced for dissociation (C.I.D.) energy, more fragment ions were observed while the response of M^+ and $[M+H]^+$ lowered significantly. When the C.I.D. energy was set at 1.5 eV, the main fragment ions at *m/z* 262 and 249 corresponding to an *N*-demethylation and an *N*-didemethylation from DMS showed a highest MS response. Meanwhile, the most abundant product ion from the I.S. was *m/z* 247. Additional tuning of the ESI source parameters such as the capillary temperature, the flows of sheath gas and auxiliary gas (N_2) and the spray voltage onto the transitions *m/z* 278 > 262 (DMS) and *m/z* 263 > 247 (I.S.) further improved the sensitivity.

3.2.1. Apparent recovery

The apparent recovery of the analytical method was measured for both DMS and the I.S. The mean extraction apparent recoveries expressed at the 95% interval of confidence were (94.2–104.8%) for DMS and (92.3–106.3%) for the I.S. Such results were indicative of the absence of co-precipitation of the analytes with proteins.

3.2.2. Limits of detection (LOD) and quantification (LOQ)

Two criteria were used to define LOQ, *i.e.*, (1) the analytical reponse at LOQ must be five times the baseline noise and (2) the analytical reponse at LOQ can be detected with sufficient precision (10–15%) and accuracy (80–120%) [8–12]. LOD is defined as the lowest concentration of DMS at which the signal is larger than three times the baseline noise [8–12]. The measured LOQ and LOD values for DMS in plasma using this method were about 0.15 and 0.05 μg L^{-1}, respectively. These results well met the requirements of quantifications of DMS in plasma that in certain cases, concentrations were lower than 2.0 μg mL^{-1}.

3.2.3. Linearity

The calibration curves were linear in the concentration range from 0.5 to 50.0 μg L^{-1}. The linearity (R^2) was better than 0.999. A statistical test of linearity was performed for each curve using a non-weighed analysis of variance. The test showed an excellent linearity at 0.01 significant level ($p > 0.5$ for all curves).

Table 1
Results on precision and accuracy of the HPLC assay using plasma samples

Variation inter-day ($n=9$)		
Spiked DMS concentrations (μg L^{-1})	Mean measured concentrations (μg L^{-1})	CV (%)
Calibration curves		
0.5	0.63	10.55
5	5.23	7.60
10	10.26	9.98
20	20.15	7.85
30	30.58	5.79
40	40.02	4.01
50	49.44	2.87
Variation intra-day ($n=6$)		
Quality controls		
5	5.29	5.48
20	19.92	8.36
40	38.98	4.55
Accuracy ($n=9$)		
Spiked DMS concentrations (μg L^{-1})	95 % interval of confidence	
Calibration curves		
0.5	86.51–113.49	
5	92.29–108.98	
10	90.36–109.65	
20	92.92–110.01	
30	91.09–106.25	
40	97.09–104.22	
50	98.06–103.14	
Calibration graph	$Y = 0.0408x + 0.0062$	
Mean correlation coefficient (R^2)	0.999	

3.2.4. Precision and accuracy

The intra-day precision (expressed by the coefficient of variation of replicate analyses) was estimated on the three quality control levels and the inter-day precision on the seven calibration standard levels. Table 1 shows the results obtained for the intra-assay (variation intra-day) and inter-assay (variation inter-day) precision for DMS. The precision for DMS under investigation

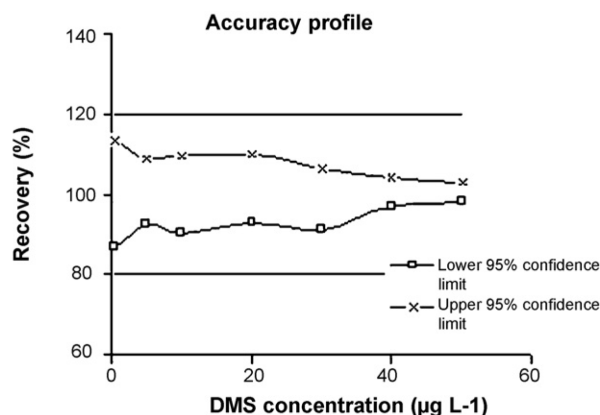

Fig. 3. Accuracy profile of DMS.

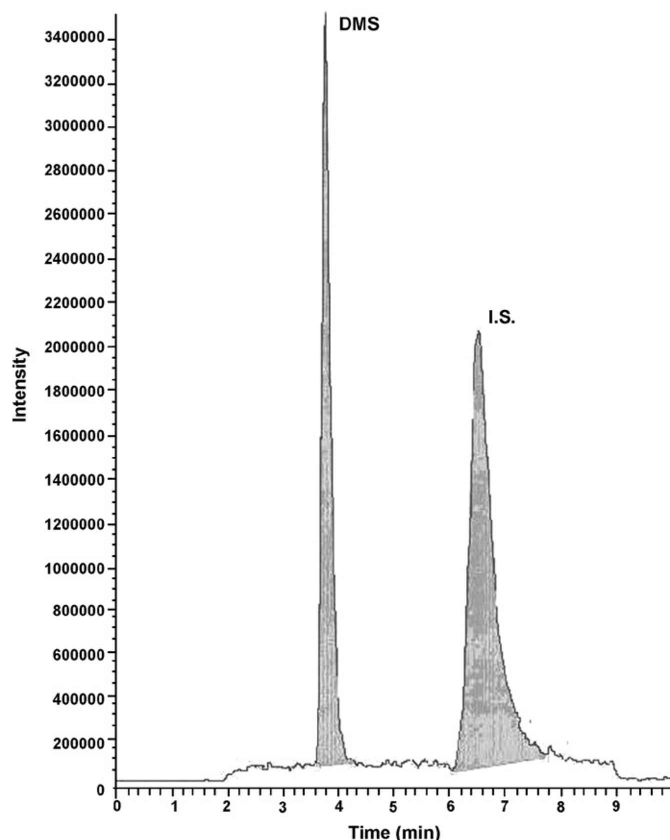

Fig. 4. Analysis of 250 μL plasma issued from the analysis of a blood sample collected on a hospitalised infant treated by oral administration dose of 10 mg of DMS, 1 h before the next administration.

at reported concentrations well met the requirements of validation.

The accuracy (expressed by the ratio between measured and theoretical concentration) was evaluated between 0.5 and 50 $\mu g\,L^{-1}$ of DMS. Informations of the accuracy profile of this analytical method is reported in Table 1. These values showed that all points are accurately quantified in the dynamic range of 0.5–50.0 $\mu g\,L^{-1}$ (Fig. 3).

The analytical validation showed that quantification of DMS in plasma could be accurately determined with acceptable precision inside the concentration range of 0.5–50.0 $\mu g\,L^{-1}$.

3.2.5. Clinical application

The validated method presented is being applied to plasma samples collected at the Paris hospitals in France allowing therapeutic monitoring of DMS. Fig. 4 shows a representative chromatogram issued from a taking of blood on a hospitalised infant treated by oral administration dose of 10 mg of DMS.

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