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ORIGINAL ARTICLE

Spectrophotometric determination of the sulfhydryl containing drug mesna

Rim S. Haggag [a], Dina A. Gawad [a], Saeid F. Belal [a], Hadil M. Elbardisy [b,*]

[a] *Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, 21521 Alexandria, Egypt*
[b] *Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Damanhour, 21532 Damanhour, Egypt*

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Abstract Four simple and sensitive spectrophotometric methods were developed for the determination of the sulfhydryl containing drug mesna (MSN). Methods I and II rely on nucleophilic aromatic substitution reactions using two UV tagging reagents namely: 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) for method I and 2,4-dinitrofluorobenzene (DNFB) for method II. Both reactions took place in alkaline buffered medium and the obtained yellowish products were measured at 414 and 332 nm for methods I and II, respectively. Methods III and IV are indirect spectrophotometric methods based on the suppressive effect of MSN on the absorption of two ternary complex systems which are composed of 1,10-phenanthroline, silver and eosin for method III and 1,10-phenanthroline, silver and bromopyrogallol red for method IV. The decrease in absorbance of the ternary complexes was measured at 547 and 635 nm for methods III and IV, respectively. All the experimental parameters affecting these reactions were carefully studied and optimized. The methods were validated as per the ICH guidelines. The methods were applicable in the linearity ranges 4–18 μg/mL for method I, 4–16 μg/mL for method II, 0.25–2.25 μg/mL for method III and 0.25–1.75 μg/mL for method IV. The proposed methods were successfully applied for the analysis of MSN in its commercial ampoules and no interference was encountered from the present excipients as indicated by the satisfactory percentage recoveries. The results obtained were in a good agreement with those obtained from a previously published method of the investigated drug.

1. Introduction

Mesna (MSN; Fig. 1) is chemically known as sodium 2-sulfanylethanesulfonate.[1] It is an antioxidant used for the prevention of urothelial toxicity in patients being treated with the antineoplastics ifosfamide or cyclophosphamide. In the kidney, dimesna, the inactive metabolite of mesna, is reduced to free mesna. This has thiol group that reacts with the metabolites of ifosfamide and cyclophosphamide, including acrolein, which are considered to be responsible for the toxic

* Corresponding author at: Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Damanhour, 21532 Damanhour, Egypt. Tel.: +20 100 754 4874; fax: +20 453334596.
E-mail address: Hadil.mamdouh@yahoo.com (H.M. Elbardisy).
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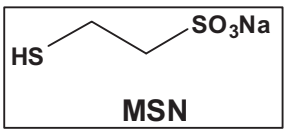


Figure 1 Chemical structure of mesna (MSN).

effects on the bladder. MSN is also used as a mucolytic in the management of some respiratory-tract disorders.[2] Besides these indications, MSN was found to be effective in the treatment of intestinal inflammation probably by scavenging reactive oxygen species.[3] Moreover, recent studies on MSN have shown that its antioxidant and antifibrotic properties can be of potential therapeutic value in protecting the liver against fibrosis and oxidative injury due to biliary obstruction.[4] Because of the increased interest in using MSN for treating various disorders, it was assumed that its quantitative determination would be of significant importance in clinical practice. MSN is official in both BP 2013[1] and USP 34,[5] both describe an indirect iodimetric titration method for the determination of the drug in its pure powder form. Screening the literature revealed various techniques that were used for MSN quantitation. Of these we can mention: a kinetic spectrofluorimetric method using cerium(IV) as an oxidizing agent,[6] and different spectrophotometric methods utilizing several reagents such as potassium permanganate,[7] methyl orange and congo red,[8] N,N-dimethyl-p-phenylenediamine[9] and ferric solution.[10] Other techniques include high performance liquid chromatography (HPLC),[11–13] capillary electrophoresis (CE)[12] and Raman spectroscopy.[14] In biological fluids, most of the methods published for MSN quantitation were based on HPLC.[15–17]

The aim of this work was to develop simple, sensitive, reliable and inexpensive spectrophotometric methods for MSN quantitation in bulk form and in its commercial ampoules. Methods I and II depend on the reaction of MSN with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and 2,4-dinitrofluorobenzene (DNFB) in borate buffer, to yield yellowish colored condensation products which were measured at their λ_{max}. Methods III and IV involve two indirect spectrophotometric methods based on the suppressive effect of MSN on the absorbance of two ternary complexes: 1,10-phenanthroline, silver and eosin $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{TBF}^{2-}$ for method III and 1,10-phenanthroline, silver and bromopyrogallol red $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{BPR}^{2-}$ for method IV. The decrease in absorbance of the ternary complexes was found to be proportional to the drug concentration. All the experimental conditions affecting these reactions were studied and optimized.

2. Experimental

2.1. Instrumentation

- Specord S600 spectrophotometer, associated with WinAspect software version 2.3, Analytik Jena AG, Germany. A 1-cm quartz cell (Analytik Jena) was used.
- A thermostatically controlled water bath, Köttermann, Germany.
- J.P. SELECTA, S.A. sonicator, Spain.
- Digital pH meter 3310 Jenway.

2.2. Materials and reagents

- All solvents, chemicals and reagents were of pure analytical grade.
- Pharmaceutical grade of MSN was purchased from Fluka (Sigma Aldrich, St. Louis, USA). It was certified to contain 99.04%.
- 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) 98%, (purchased from Sigma, St. Louis, USA) was prepared as 0.1% w/v solution in methanol. The reagent was stable for two weeks if kept under refrigeration.
- 2,4-Dinitrofluorobenzene (DNFB) (Hopkin and Williams Co., Essex-UK) was prepared as 0.2% w/v solution in methanol. The reagent was freshly prepared and protected from light.
- Silver nitrate, assay >99% (Fluka, packed in Switzerland) was used as 5×10^{-4} M solution in double distilled water and it was protected from light.
- 1,10-Phenanthroline (Prolabo) was prepared as 1×10^{-3} M solution in warm distilled water.
- Eosin (TBF) (2,4,5,7-tetrabromofluorescein) (Riedel-De Haën AG) was prepared as 1×10^{-4} M aqueous solution.
- Bromopyrogallol red (BPR) (Aldrich Chem. Co.). Its solution was prepared by dissolving 25 mg of BPR in 100 mL of 1% ammonium acetate. It should be freshly prepared.
- Ethylenediaminetetraacetic acid (EDTA) (Chemajet, Alexandria, Egypt) was prepared as 0.1 M aqueous solution.
- Gelatin (Veb Laborchemie Apolda, Germany) was prepared as 0.5 g% solution in warm distilled water.
- Borate buffer (disodium tetraborate) (Gateway Co., Egypt) was prepared as 0.05 M aqueous solution.
- Boric acid (Gomhouria Co., Alexandria, Egypt) was prepared as 0.1 M solution in warm distilled water.
- Sodium acetate (El. Nasr Pharmaceutical Chemicals Co. Egypt) was prepared as 1 M aqueous solution.
- NaOH (FEMICO Co., Alexandria, Egypt) was prepared as 0.1 and 1 M aqueous solutions.
- Acetic acid (El. Nasr Pharmaceutical Chemicals Co. Egypt) was prepared as 10% aqueous solution.
- Methanol (SDFCL, Mumbai, India).
- Fresh double distilled water was used.

2.3. Pharmaceutical preparation

Uromitexan® ampoules (Batch No. 3A109A) are manufactured by Baxter oncology GmbH, Germany. The ampoules are labeled to contain 400 mg mesna/4 mL ampoule.

2.4. Standard stock solutions

For methods I and II, a stock solution of MSN was prepared as 20 mg% in methanol. While for methods III and IV, it was prepared as 12.5 mg% solution in methanol then it was diluted to obtain a working solution of final concentration 1.25 mg%. These stock solutions were stored in the refrigerator at 4 °C.

2.5. General procedure and construction of the calibration curves

2.5.1. Methods I and II

Accurate volumes from MSN standard stock solution (20 mg %) (0.2–0.9 mL) for method I and (0.2–0.8 mL) for method II (in 0.1 mL increments) were transferred into two sets of 10 mL volumetric flasks. The specified volume of borate buffer of the optimum pH was added, followed by the appropriate volume of NBD-Cl (Method I) or DNFB (Method II) (Table 1). The reaction mixtures were mixed and left to stand for 30 min at room temperature. The solutions were completed to the mark with methanol and the absorbances were measured at 414 nm (Method I) and 332 nm (Method II) against a similarly treated blank. The calibration graphs were constructed by plotting the absorbance values against their corresponding concentrations.

2.5.2. Methods III and IV

Into two sets of 25 mL volumetric flasks different aliquots of the standard working solution of MSN (1.25 mg%) (0.5–4.5 mL) for method III and (0.5–3.5) for method IV (in 0.5 mL increments) were transferred. The appropriate volumes of the following reagents were successively added with mixing: silver nitrate (5×10^{-4} M), EDTA (0.1 M), gelatin (0.5 g%), 1,10-phenanthroline (1×10^{-3} M), sodium acetate of the specified pH (1 M) and finally TBF (1×10^{-4} M) for method III or BPR for method IV (Table 2). The solutions were mixed, allowed to stand at room temperature for ten minutes and then the flasks were made up to 25 mL with distilled water. The absorbance values were measured at 547 nm (for Method III) and at 635 nm (for Method IV) and subtracted from the corresponding reading of a similarly treated blank. The decrease in absorbance was plotted as a function of concentration of the drug to construct the calibration curves.

Table 1 Assay parameters for the determination of MSN using the proposed methods I and II.

Parameters	Method I	Method II
Borate buffer pH	8.5	9.5
Borate buffer volume (mL)	0.7	0.5
Reagent volume (mL)	0.8	0.4
Reaction temperature (°C)	Room temperature	
Reaction time (min)	30	

Table 2 Assay parameters for the determination of MSN using the proposed methods III and IV.

Parameters	Method III	Method IV
Acetate buffer pH	7	6.6
Acetate buffer volume (mL)	0.4	0.6
Silver nitrate volume (mL)	1.6	1.2
EDTA volume (mL)	1	1
Gelatin volume (mL)	2	4
1,10-Phenanthroline volume (mL)	0.6	0.5
TBF volume (mL)	3.5	—
BPR volume (mL)	—	0.6
Reaction time (min)	10	
Reaction temperature (°C)	Room temperature	

2.6. Procedure for ampoules

2.6.1. Uromitexan® ampoule assay: (400 mg mesna/4 mL ampoule)

The contents of five ampoules were mixed well and an aliquot of 1 mL was accurately transferred into a 100 mL volumetric flask, the volume was completed to the mark with methanol and the solution was thoroughly mixed to obtain a solution of concentration 100 mg%. For methods I and II, dilution was made by transferring 10 mL from the latter solution into another 50 mL volumetric flask where the volume was adjusted with methanol. Then, different appropriate aliquots of this solution were assayed as described under "General procedure and construction of the calibration curves" (Section 2.5.1). For methods III and IV, dilution was done by transferring 12.5 mL from the 100 mg% solution to a 100 mL volumetric flask and completing to the mark with methanol. The latter solution was further diluted 10 folds with methanol to obtain a test solution of concentration 1.25 mg%. Different appropriate aliquots of this diluted solution were then assayed as specified under "General procedure and construction of the calibration curves" (Section 2.5.2).

3. Results and discussion

MSN is a non UV absorbing compound that lacks any chromophore in its structure, therefore two strategies were adopted to render its spectrophotometric analysis feasible: first, by derivatization with chromogenic reagents, or second, by suppressing the absorbance of silver containing ternary complexes upon the reaction of –SH group in MSN with silver ion.

3.1. Methods I and II

In methods I and II, MSN reacted with either NBD-Cl or DNFB in aqueous alkaline buffered medium through nucleophilic aromatic substitution reaction to yield yellowish condensation products that absorb maximally at 414 and 332 nm, respectively (Figs. 2 and 3). The following schemes present the proposed pathways for the reaction of MSN with NBD-Cl and DNFB (see Schemes 1 and 2).

3.1.1. Reaction stoichiometry

Job's method of continuous variation was applied to determine the reaction stoichiometry between MSN and the two reagents NBD-Cl and DNFB. This was done by preparing equimolar concentrations of the drug solution and the two reagents (1.2×10^{-3} M) and adding different volumes of the drug and each reagent to obtain different ratios of total volume of 1 mL. From Job's method plots we can conclude that the reaction ratio between MSN/NBD-Cl or between MSN/DNFB is (1:1) (Fig. 4).

3.1.2. Optimization of the reaction conditions

The reaction conditions were studied through a series of experiments to determine the optimum experimental values that give maximum and reproducible measurements. The optimized reaction conditions were summarized in Table 1.

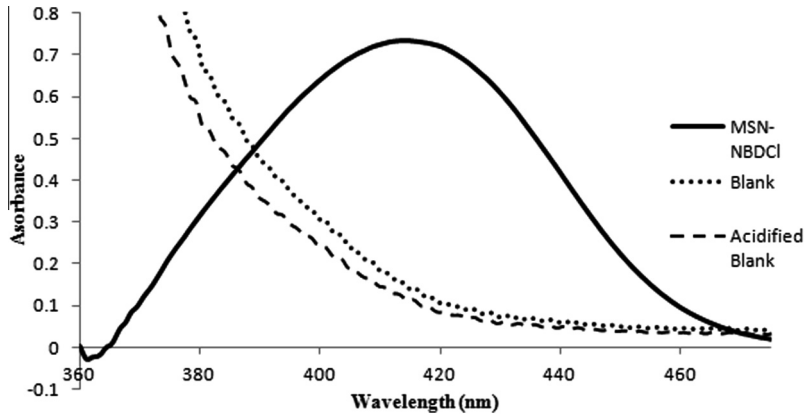


Figure 2 Absorption spectra of the derivative formed between NBD-Cl and 8 μg/mL MSN (—), NBD-Cl blank solution (. . ..) and NBD-Cl blank solution acidified with 0.2 mL of 5 M HCl (- - -).

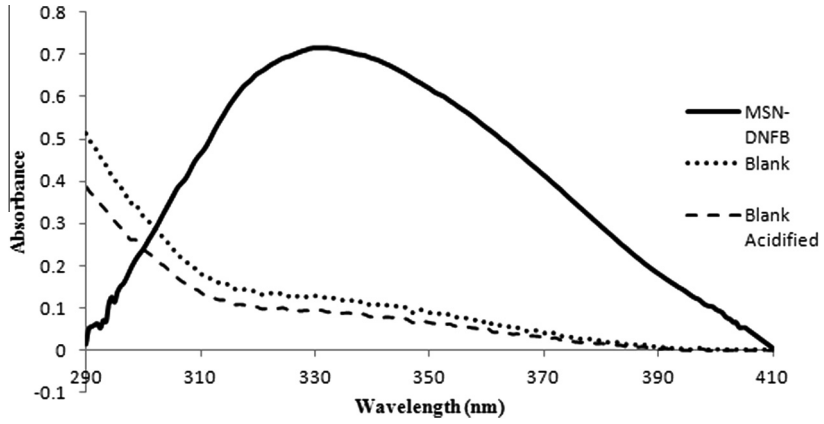
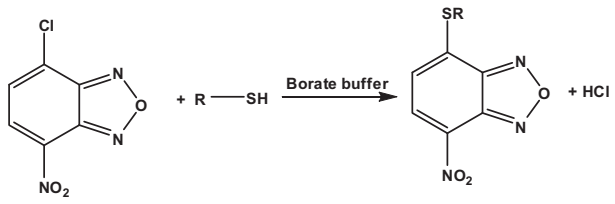
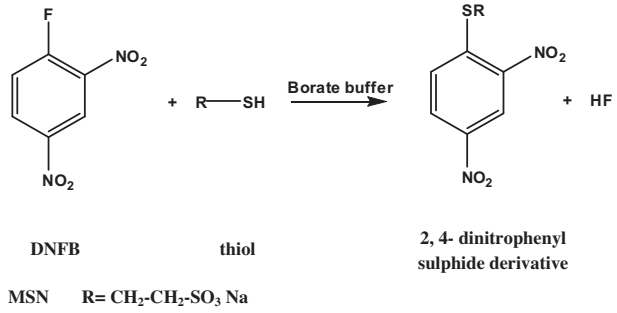


Figure 3 Absorption spectra of the derivative formed between DNFB and 12 μg/mL MSN (—), DNFB blank solution (. . ..) and DNFB blank solution acidified with 0.2 mL of 5 M HCl (- - -).



Scheme 1 Proposed pathway for the reaction of MSN with NBD-Cl.



Scheme 2 Proposed pathway for the reaction of MSN with DNFB.

3.1.2.1. Effect of buffer pH. The effect of borate buffer pH on the nucleophilic substitution reactions was studied over the pH range 7–10.5 (at 0.5 pH unit interval) while keeping all other parameters constant. In method I, it was found that the absorbance intensity of the formed derivative increased slightly at pH 7.5 and further increase in the buffer pH had no significant effect on the product response (Fig. 5). So pH 8.5 was chosen for this study. In method II, it is obvious from the same figure that below pH 8.5 the formed product had a poor response, but by gradual increase in the buffer pH, the response increased and reached its maximum value at pH range 8.5–10 then it started to decrease. Therefore, pH 9.5 was chosen for this method. Other buffers having the same optimum pH value such as bicarbonate or phosphate buffers were tried and compared with 0.05 M borate buffer which proved to be superior to other buffers having the same pH value owing to the higher absorbance readings obtained in borate buffer in both methods I and II.

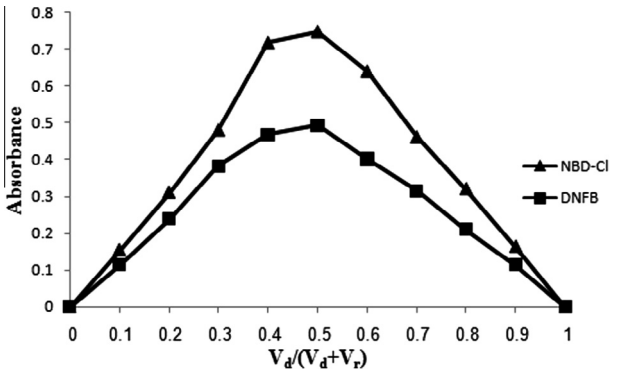


Figure 4 Job's method of continuous variation plot for the determination of the stoichiometric ratio between MSN with NBD-Cl and DNFB.

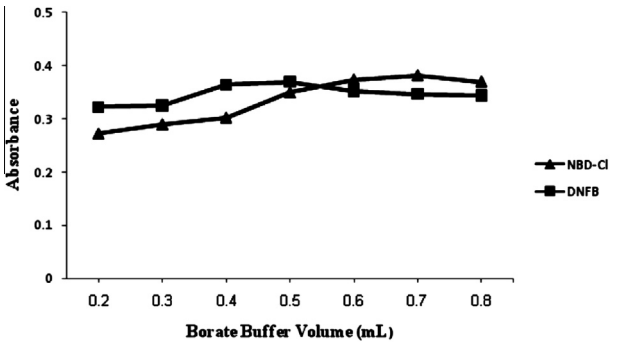


Figure 6 Effect of borate buffer volume on the substitution reaction of 4 and 6 μg/mL MSN with NBD-Cl and DNFB, respectively.

3.1.2.2. Effect of buffer volume. The effect of borate buffer volume on the absorbance intensity of the formed products was studied. This was done using different volumes of borate buffer (0.2–0.8 mL) of the optimized pH while keeping all other factors constant. Fig. 6 shows that the highest absorptions were achieved using 0.7 and 0.5 mL borate buffer for methods I and II, respectively.

3.1.2.3. Effect of NBD-Cl and DNFB volume. The effect of NBD-Cl and DNFB concentrations on the color development was investigated by trying different volumes from the reagent stock solution ranging from 0.2 to 1 mL (in 0.2 mL increments). In method I, it was found that using less than 0.4 mL of this reagent resulted in lower absorption intensity of the formed derivative. A volume of 0.8 mL of 0.1% w/v NBD-Cl was chosen as the most appropriate volume (Fig. 7). For Method II, it was found that 0.4 mL of 0.2% w/v DNFB were suitable for the reaction; as further increase in the reagent volume resulted in a gradual decrease in the product response (Fig. 7).

3.1.2.4. Effect of reaction time and temperature. The effect of reaction temperature and time on the nucleophilic substitution reactions was studied for both methods I and II. This was performed by investigating the effect of four different temperatures (room temperature, 40, 60 and 80 °C) at constant time interval (every 10 min). It was found that the reactions proceeded at room temperature and attained their maximum sensitivity after 30 min, whereas heating resulted in non reproducible results and much lower sensitivity (Table 3).

3.1.2.5. Effect of acidification using 5 M HCl. It was reported that heating NBD-Cl in aqueous methanolic buffer resulted in the appearance of two absorption bands with maxima at 372 and 462 nm. These two bands correspond to the formation of NBD-OCH_3 and its hydrolysis product NBD-OH. Acidification of a solution containing NBD-OH results in a hypsochromic shift of its absorption band from 462 to 387 nm. This shift was attributed to the protonation of the dissociated hydroxyl group of NBD-OH.[18] Thus it was concluded that heating NBD-Cl is the main reason of appearance of these products and the increase in the background readings. In our study, MSN reacted with NBD-Cl at room temperature, thus the acidification step was of no need.

For DNFB, it has been reported that the excess reagent must be hydrolyzed to 2,4-dinitrophenol by acidification to get rid of the excess reagent spectral interference.[19] But in some published methods the acidification step was omitted, as it did not affect the absorbance significantly.[20] In our study, we investigated the effect of acidification by comparing the absorbance value of the blank solution with and without acidification at 332 nm (λ_{max} of the reaction product; Fig. 3). It is clear that acidification led to a small decrease in the blank readings, which could be easily eliminated through the instrumental background correction (auto zero of blank); so the

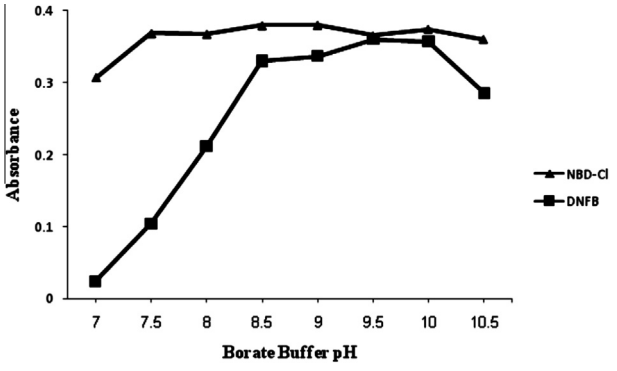


Figure 5 Effect of borate buffer pH on the substitution reaction of 4 and 6 μg/mL MSN with NBD-Cl and DNFB, respectively.

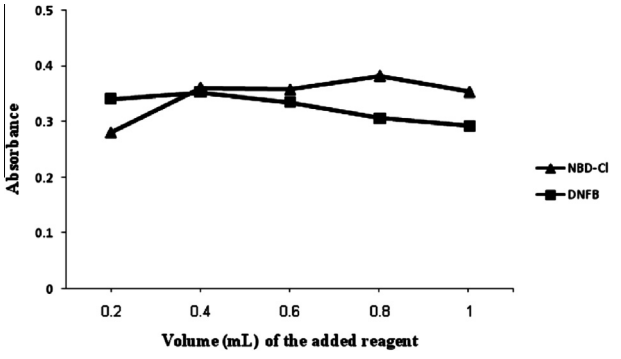


Figure 7 Effect of NBD-Cl and DNFB volumes on their substitution reaction with 4 and 6 μg/mL MSN, respectively.

Table 3 Effect of reaction temperature and time on the absorbance of the products obtained from the reactions of NBD-Cl and DNFB with MSN in borate buffer medium.

Time (min)	Temperature (°C)							
	Method I				Method II			
	Room temperature	40 °C	60 °C	80 °C	Room temperature	40 °C	60 °C	80 °C
10	0.335	0.330	0.329	0.329	0.338	0.289	0.257	0.250
20	0.354	0.325	0.341	0.315	0.346	0.233	0.249	0.227
30	0.367	0.333	0.316	0.333	0.370	0.309	0.260	0.264
40	0.363	0.348	0.324	0.290	0.369	0.235	0.220	0.248

acidification step has been omitted to render the procedure simpler.

3.1.2.6. Stability of the colored product. It was found that the color of the formed derivatives was stable for at least 60 min.

3.2. Methods III and IV

The use of ternary complexes in quantitative analysis is widespread. A large number of possible combinations between reagents are capable of forming different ternary complex systems. These complexes can be utilized in various analytical fields; spectrophotometry[21,22] and fluorimetry.[23,24] In the present work, two ternary complexes were chosen for the quantitation of MSN namely: 1,10-phenanthroline, silver and eosin [(phen-Ag-phen)$^{+}$]$_2$·TBF^{2-} (Method III) and 1,10-phenanthroline, silver and bromopyrogallol red [(phen-Ag-phen)$^{+}$]$_2$·BPR^{2-} (Method IV).

When increasing amounts of the sulfhydryl containing drug were added to each complex, a proportional decrease in the color of the complexes was observed. The reason for this is that the –SH group in MSN reacted with silver ion present in the complexes causing the fixation of silver as silver mercaptide. As a result, the concentration of silver ion available for the formation of the ternary complexes decreased. This causes a concomitant decrease in the absorbance of the solutions which is proportional to MSN concentration.[25] Figs. 8 and 9 show the absorption spectra of the two ternary complexes formed in the absence and in the presence of MSN. The reaction mechanisms are proposed in Scheme 3.

As these spectrophotometric methods depend on the formation of ternary complexes, they are subjected to interference from foreign ions: ions which would interfere by reacting with BPR, with TBF, with 1,10-phenanthroline or with all of them. For this reason 1 mL of 0.1 M EDTA solution was added to all samples to suppress the interference of any extraneous ions. The reaction of MSN with the two ternary complexes is not affected by the presence of EDTA. Therefore, it could be possible to determine MSN in the presence of very large excess of interfering ions provided that they form reasonably stable EDTA complexes at pH 7 and 6.6, and these complexes don't absorb appreciably at the wavelengths of detection.[26–28]

3.2.1. Optimization of the reaction conditions

The different experimental parameters were optimized in order to achieve maximum suppressive effect of MSN on the absorbance of the two ternary complexes. The optimum conditions are summarized in Table 2.

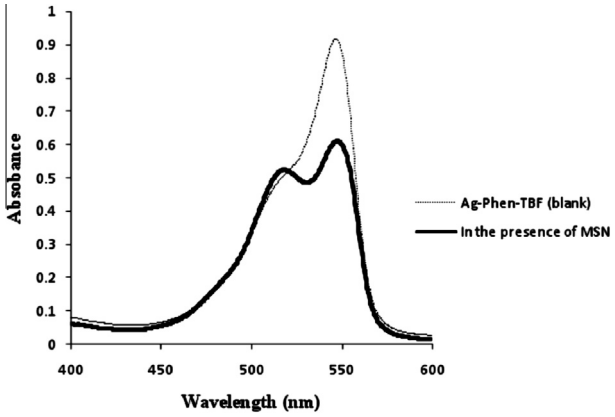


Figure 8 Absorption spectrum of the ternary complex [(phen-Ag-phen)$^{+}$]$_2$·TBF^{2-} solution in the absence (……) and in the presence of 1 μg/mL MSN (——).

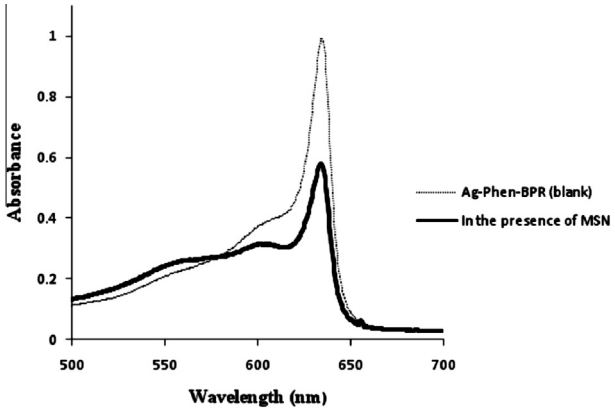
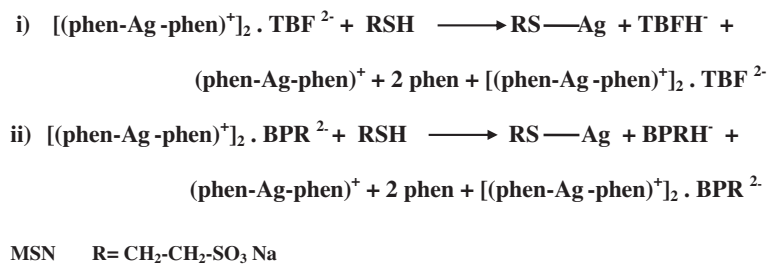


Figure 9 Absorption spectrum of ternary complex [(phen-Ag-phen)$^{+}$]$_2$·BPR^{2-} solution in the absence (……) and in the presence of 0.7 μg/mL MSN (——).

3.2.1.1. Effect of buffer pH. In many previous reports it was found that the maximum suppressive effect of the drugs on the absorbance of these ternary complexes is attained at pH range 6–8.[25,29,30] In addition, using more alkaline buffers increases the incidence of BPR oxidation which becomes appreciable at pH > 9.[26] So the inhibition effect of MSN was investigated within this pH range. The best results were obtained using acetate buffer of pH 7 and 6.6 for methods III and IV, respectively (Fig. 10).



Scheme 3 Proposed pathway for the reaction of MSN with the two ternary complexes.

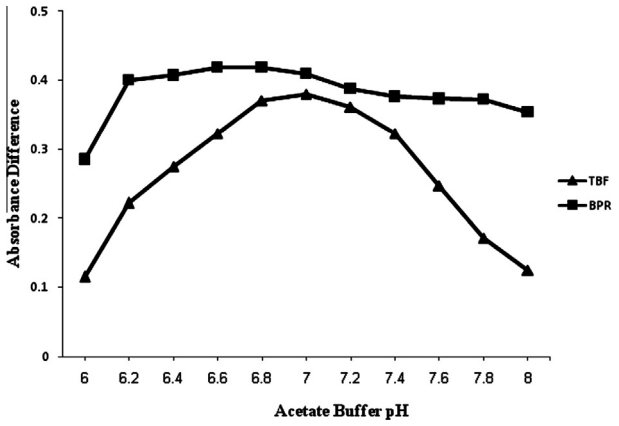


Figure 10 Effect of buffer pH on the reaction of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}TBF^{2-}$ and $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$ with 1.2 and 0.7 μg/mL MSN, respectively.

3.2.1.2. Effect of buffer volume. The effect of acetate buffer volume on the decrease in blank absorbance was studied. The buffer volume was varied from 0.2 to 1 mL (in 0.2 mL increments) while keeping all other parameters constant. The study revealed that the maximum absorption subtraction signal is reached when using 0.4 and 0.6 mL of acetate buffer for methods III and IV, respectively (Fig. 11).

3.2.1.3. Effect of gelatin volume. The ternary complex color systems are unstable (owing to their colloidal nature), a pink precipitate (in case of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}TBF^{2-}$) or blue precipitate (in case of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$) settles down on standing. To solve this problem, we found in literature different stabilizing agents were tried such as: triton X-100, sodium lauryl sulphate, polyvinyl alcohol and sodium carboxymethylcellulose. The best results were obtained using gelatin,[27,30] it acts as a protective colloid; which solubilizes the precipitate and produces a clear solution of the two complexes. Moreover, gelatin has to be added before introducing the dye solutions (TBF or BPR) to obtain more reproducible results.

The effect of gelatin volume on the measured signal was investigated. In case of method III, the optimum gelatin volume was found to be 2 mL. Whereas, by increasing the gelatin volume beyond 3 mL the measured signal started to decrease (Fig. 12). A volume of 4 mL of gelatin was found to be suitable for the reaction of MSN with the ternary complex $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$ (Method IV), as the measured signal was poor using gelatin volumes less than 2 mL (Fig. 12).

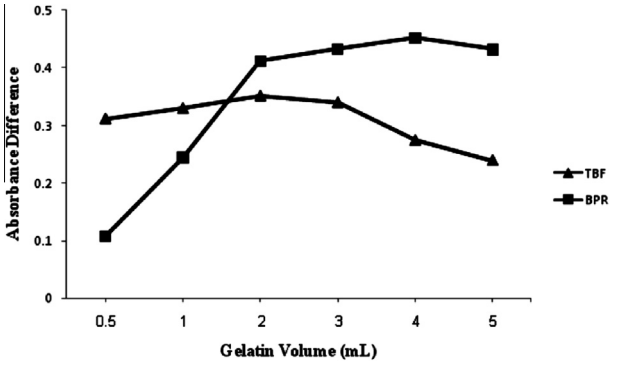


Figure 12 Effect of gelatin volume on the reaction of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}TBF^{2-}$ and $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$ with 1.2 and 0.7 μg/mL MSN, respectively.

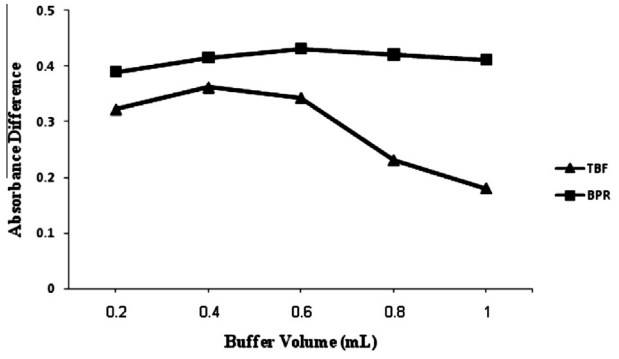


Figure 11 Effect of buffer volume on the reaction of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}TBF^{2-}$ and $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$ with 1.2 and 0.7 μg/mL MSN, respectively.

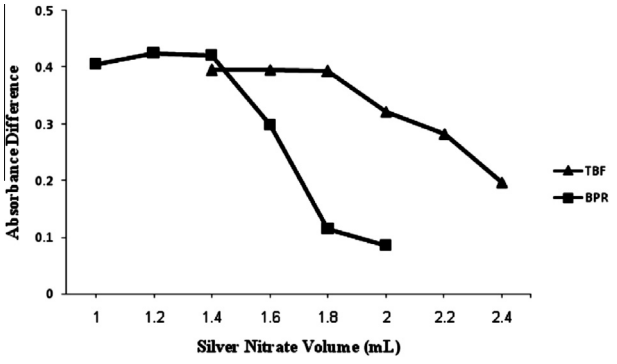


Figure 13 Effect of silver nitrate volume on the reaction of $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}TBF^{2-}$ and $[(phen\text{-}Ag\text{-}phen)^{+}]_2{\cdot}BPR^{2-}$ with 1.2 and 0.7 μg/mL MSN, respectively.

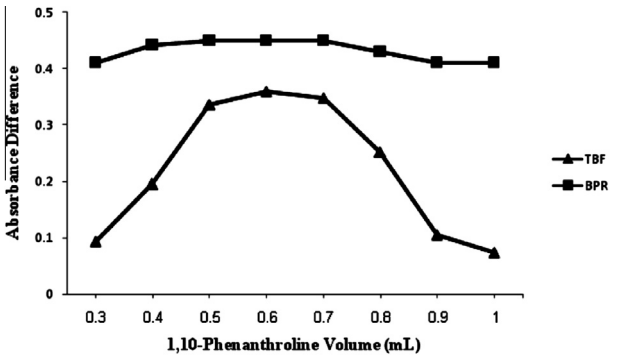


Figure 14 Effect of phenanthroline volume on the reaction of $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{TBF}^{2-}$ and $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{BPR}^{2-}$ with 1.2 and 0.7 μg/mL MSN, respectively.

3.2.1.4. Effect of silver nitrate volume. Silver nitrate concentration is an important factor in our study. Its volume was varied from 1.4 to 2.4 mL (Method III) and from 1 to 2 mL (Method IV), in 0.2 mL increments while keeping all other parameters constant. It was found that 1.6 and 1.2 mL of silver nitrate were the optimal volumes for the reaction of MSN with $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{TBF}^{2-}$ and $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{BPR}^{2-}$, respectively (Fig. 13).

3.2.1.5. Effect of 1,10-phenanthroline concentration. Phenanthroline participates in the constitution of the two ternary complexes. Thus studying the effect of its concentration on the absorption subtraction signal was of considerable importance. The volume of 1,10-phenanthroline was varied from 0.3 to 1 mL (in 0.1 mL increments) and it was found that 0.6 and 0.5 mL of this reagent are suitable for method III and method IV, respectively (Fig. 14).

3.2.1.6. Effect of TBF and BPR volumes. The effect of TBF and BPR volumes was studied in order to obtain maximum and reasonable blank readings. TBF concentration was studied by varying its volume from 2 to 5 mL in 0.5 mL increments. It was found that the maximum blank reading was reached after the addition of 3 mL and further addition of TBF gave a plateau. Therefore, three and half milliliters of TBF were chosen for method III (Fig. 15). BPR concentration was also studied and its volume was varied from 0.1 to 0.9 mL in 0.1 mL increments. As shown in Fig. 16, the most suitable BPR volume was 0.6 mL and further increase in its volume did not affect the absorbance readings.

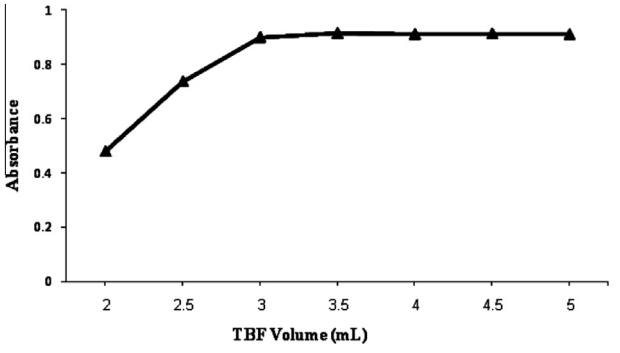


Figure 15 Effect of TBF volume on the formation of the ternary complex $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{TBF}^{2-}$.

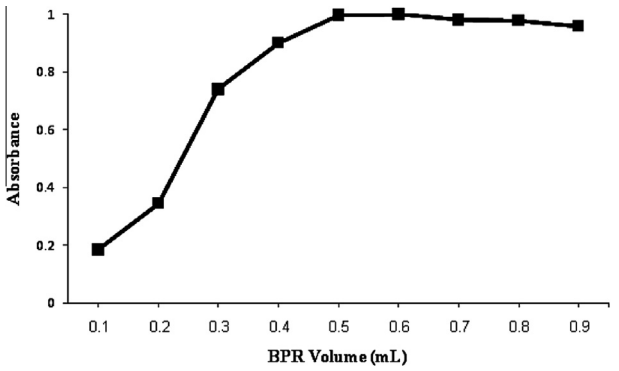


Figure 16 Effect of BPR volume on the formation of the ternary complex $[(\text{phen-Ag-phen})^{+}]_2{\cdot}\text{BPR}^{2-}$.

3.2.1.7. Effect of reaction time. The suppressive effect of MSN on the formation of the two ternary complexes was investigated as a function of time. The solutions were allowed to stand at room temperature for 70 min and the signal was measured every 5 min through this time interval. It was found that the reaction between MSN and Ag^{+} was completed within 10 min. The absorbance difference remained constant for at least 1 h.

3.3. Validation of the proposed methods

The proposed spectrophotometric methods were validated as per the International Conference on Harmonization (ICH) guidelines on validation of analytical procedures.[31]

3.3.1. Linearity and concentration ranges

The linearity of the proposed spectrophotometric methods was evaluated by analyzing a series of different concentrations of the drug with concentration ranges stated in Table 4. The calibration curves for methods I and II were plotted using the absorbance intensity of the formed derivatives against the corresponding concentration of MSN. While for methods III and IV, calibration curves were constructed as a function of a decrease in absorbance of the ternary complexes against the increasing concentration of the drug. A minimum of five concentrations is recommended for establishing the calibration curve. A straight line was obtained in each case. The obtained results were subjected to statistical analysis using the least squares method for calculating correlation coefficients, intercepts, slopes, standard deviation of intercept (S_a) and slope (S_b) and standard deviation of residuals ($S_{y/x}$). All these validation parameters are summarized in Table 4.

The regression data showed the adherence of the system to Beer's law and this was indicated by the high values of correlation coefficients ($r > 0.999$) and the small values of the intercepts. In addition, deviation around the slopes could be further calculated by the RSD% of the slopes (S_b%) which were found to be less than 2%. Linearity can be further guaranteed by the analysis of variance (ANOVA) test. The *F*-value is considered as the most important statistical tool in this test, it is defined as the ratio of the mean of squares due to regression divided by the mean of squares due to residuals. The high *F* values reveal an increase in the mean of squares due to regression and a decrease in the mean of squares due to residuals.

Table 4 Regression and statistical parameters for the determination of MSN using the proposed methods.

Parameters	Method			
	Method I	Method II	Method III	Method IV
λ_{max} (nm)	414	332	547	635
Concentration range (μg/mL)	4–18	4–16	0.25–2.25	0.25–1.75
Regression equation				
Intercept (a)	2.89×10^{-2}	2.71×10^{-3}	-2.14×10^{-2}	-1.45×10^{-1}
Slope (b)	8.78×10^{-2}	6.08×10^{-2}	3.3×10^{-1}	8.31×10^{-1}
Correlation coefficient (r)	0.9994	0.9994	0.9998	0.9998
S_a[a]	1.42×10^{-2}	1.36×10^{-2}	4.31×10^{-3}	7.73×10^{-3}
S_b[b]	1.19×10^{-3}	1.14×10^{-3}	3×10^{-3}	7.06×10^{-3}
$S_{y/x}$[c]	1.55×10^{-2}	1.05×10^{-2}	5.34×10^{-3}	9.15×10^{-3}
RSD% of the slope (S_b%)	1.36	1.88	0.91	0.85
F[d]	5410.16	2866.46	12163.97	13837.07
Significance F	4.25×10^{-10}	1.44×10^{-5}	1.64×10^{-6}	3.13×10^{-8}
LOD[e] (μg/mL)	1.03	1.06	0.07	0.08
LOQ[f] (μg/mL)	3.11	3.22	0.22	0.23

[a] Standard deviation of the intercept.
[b] Standard deviation of the slope.
[c] Standard deviation of the residuals.
[d] Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).
[e] LOD = Limit of detection.
[f] LOQ = Limit of quantitation.

The greater the mean of squares due to regression, the steeper the regression line. The smaller the mean of squares due to residuals, the lesser the scatter of the experimental points around the regression line. Consequently, regression lines with high F values (low significance F) are much better than those with lower ones. Good regression lines show high values for both (r) and (F) values.[32,33]

3.3.2. Detection and quantitation limits

The limit of detection (LOD) is the lowest concentration of the analyte that can be detected but not necessarily quantitated under the stated experimental conditions while the limit of quantitation (LOQ) is the concentration at and above which the analyte can be reliably quantitated with a previously defined level of certainty.[34] LOD and LOQ of the proposed spectrophotometric methods were calculated in accordance to the formulae given by the ICH guidelines, where LOD = 3.3 S b^{-1} and LOQ = 10 S b^{-1}, where S is the standard deviation of five blank solutions and b is the slope of the regression line of the calibration curve. The small values of LOD and LOQ presented in Table 4 indicate the sensitivity of the proposed methods.

3.3.3. Accuracy

Accuracy is defined by the International Standards Organization (ISO) as 'the closeness of agreement between a test result and the accepted reference value of the analyte'.[32] The accuracy of the proposed methods was studied by applying the reactions under the specified optimum conditions at three concentration levels of MSN using three replicate determinations for each concentration.

Accuracy was expressed as recovery% and E_r% for MSN, these values are presented in Table 5 and they confirm the good accuracy of the proposed methods.

3.3.4. Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions.[31] It includes repeatability (intra-day precision) and intermediate precision (inter-day precision).

3.3.4.1. Repeatability (intra-day precision). It expresses precision under the same operating conditions, over a short interval of time. Repeatability can be assessed using three different concentration levels of MSN which were analyzed three times within the same laboratory, using the same analyst, with the same equipment, on the same day. RSD% of the obtained results were calculated and presented in Table 5.

3.3.4.2. Intermediate precision (inter-day precision). It expresses variations within the same laboratory. Typical variations to be studied include days, analyst, equipment, etc. Intermediate precision can be tested using the three different concentration levels of MSN used in the repeatability study, these concentration levels were repeated on three different days, while keeping all other parameters constant. RSD% of the obtained data was calculated as shown in Table 5.

The percentage relative standard deviation (RSD%) values from repeatability and intermediate precision were less than 2% proving the high repeatability and precision of the developed methods for the quantitative estimation of MSN in its bulk powder form.

3.3.5. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedure parameters listed in the procedure documentation and provides an indication of its suitability during

Table 5 Intra-day and inter-day precision and accuracy for the determination of MSN using the proposed methods.

Concentration (μg/mL)				Mean % recovery ± SD[a]				RSD (%)[b]				E_r (%)[c]			
Method I	Method II	Method III	Method IV	Method I	Method II	Method III	Method IV	Method I	Method II	Method III	Method IV	Method I	Method II	Method III	Method IV
(A) Intra-day precision and accuracy															
6	6	0.4	0.5	100.80 ± 1.47	99.86 ± 0.12	99.60 ± 0.68	100.14 ± 0.53	1.46	0.12	0.68	0.53	0.80	−0.14	−0.40	0.14
10	10	0.8	1	99.83 ± 0.47	100 ± 0.17	99.81 ± 0.63	99.72 ± 0.39	0.47	0.17	0.63	0.39	−0.17	0	−0.19	−0.28
14	14	2	1.75	101.32 ± 1.60	100.03 ± 0.23	99.80 ± 0.35	100.06 ± 0.64	1.58	0.23	0.35	0.64	1.32	0.03	−0.20	0.06
(B) Inter-day precision and accuracy															
6	6	0.4	0.5	99.34 ± 0.94	100.23 ± 1.27	99.67 ± 1.01	100.04 ± 0.60	0.95	1.27	1.01	0.60	−0.66	0.23	−0.33	0.04
10	10	0.8	1	100.04 ± 0.60	100.08 ± 1.04	99.96 ± 0.24	100.46 ± 0.88	0.60	1.04	0.24	0.88	0.04	0.08	−0.04	0.46
14	14	2	1.75	100.31 ± 0.79	99.94 ± 1.14	99.17 ± 0.78	99.53 ± 0.85	0.79	1.14	0.79	0.85	0.31	−0.06	−0.83	−0.47

[a] Mean ± standard deviation for three determinations.
[b] % Relative standard deviation.
[c] % Relative error.

normal usage.[31] Robustness of methods I and II was evaluated by introducing small variations in reagent volume (±0.05 mL), reaction time (±5 min) and λ of detection (λ_{max} ± 4 nm). For methods III and IV, small deliberate variations were introduced in silver nitrate volume (±0.2 mL), gelatin volume (±0.5 mL) and reaction time (±5 min). This study demonstrated that slight intended variations in the previously mentioned parameters had no significant effect on MSN determination using the proposed methods. This was obvious from the low values of RSD% which did not exceed 2% (Table 6).

3.3.6. Selectivity and specificity

Specificity is defined as the ability to unequivocally asses the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components.[5] The specificity of the proposed spectrophotometric methods could be demonstrated by their ability to quantify MSN with good accuracy and precision without interference from co-formulated excipients as indicated by the good percentage recoveries, low values of RSD % and E_r% obtained from analyzing Uromitexan® ampoules (Table 7). The specificity of methods I and II was further confirmed by applying these reactions to the co-administered drug cyclophosphamide. It was found that cyclophosphamide gave no reaction with NBD-Cl, however, it reacted with DNFB when used in concentrations above 1 μg/mL and below this range no interference was encountered. Therefore, the reaction with NBD-Cl could be applied in clinical studies to determine MSN in blood samples of patients being treated with the antineoplastic cyclophosphamide with no interference. On the other hand, the reaction with DNFB could be also applicable but when the blood level of the antineoplastic is less than 1 μg/mL.

3.4. Stability of solutions

The methanolic stock solution of MSN was found to be stable for at least two weeks when refrigerated at 4 °C.

3.5. Assay of pharmaceutical preparations

The proposed methods were applied for the determination of MSN in commercial ampoules (Uromitexan® ampoule). The drug was extracted using the same solvent used for the preparation of the standard stock solution, and then dilution to volume was made using methanol to reach concentration levels within the specified ranges. The procedures were applied following the establishment of all the experimental conditions. Five replicate determinations were made for each method and the percentage recoveries were calculated using single point quantification or from the linear regression equations. Also, the SD, RSD% and E_r% were calculated and satisfactory results were obtained for each method and they were in a good agreement with the label claim (Table 7). The results obtained were statistically compared with those of a reported spectrophotometric method[7] (utilizing alkaline potassium permanganate) using the Student's *t*-test (for accuracy) and the variance ratio *F*-test (for precision). The results in Table 7 show that the *t*- and *F*-values were smaller than the critical values at 95%

Table 6 Evaluation of the robustness of the proposed methods for the determination of MSN.

Parameters	Mean% recovery	SD	RSD (%)	Mean% recovery	SD	RSD (%)
	Method I			Method II		
Reagent volume (±0.05 mL)	99.23	1.20	1.21	98.88	1.73	1.75
Reaction time (±5 min)	98.87	1.45	1.47	99.09	0.53	0.53
λ of detection (λ_{max} ± 4 nm)	99.85	0.52	0.52	99.34	1.19	1.20
	Method III			Method IV		
Silver nitrate volume (±0.2 mL)	100.79	0.39	0.39	100.00	0.69	0.69
Gelatin volume (±0.5 mL)	98.73	0.70	0.71	97.53	0.07	0.07
Reaction time (±5 min)	99.16	1.15	1.16	100.60	0.24	0.24

Table 7 Assay results for the determination of MSN in its pharmaceutical preparation using the proposed and reference methods.

Uromitexan® ampoule	Method I	Method II	Method III	Method IV	Reference method[7,e]
Mean% recovery ± SD[a]	101.25 ± 0.61	99.89 ± 0.70	101.60 ± 1.76	100.33 ± 1.78	100.31 ± 0.74
RSD% [b]	0.60	0.70	1.73	1.77	0.74
E_r (%) [c]	1.25	−0.11	1.60	0.33	0.31
t [d]	2.20	0.92	1.51	0.03	—
F [d]	1.44	1.12	5.74	5.88	—

[a] Mean% recovery ± SD of five determinations.
[b] % Relative standard deviation.
[c] % Relative error.
[d] Theoretical values for t-test and F-test are 2.31 and 6.39, respectively at 95% confidence limit.
[e] Kinetic spectrophotometric determination of mesna using alkaline potassium permanganate.[7]

confidence level, indicating that there were no significant differences between the proposed methods and the reported one regarding accuracy and precision.

By comparing the four proposed methods with each other we can conclude that methods I and II are simpler, need less experimental work regarding reagent preparation and procedure, in addition, their selectivity was proven by analyzing MSN in the presence of the co-administered drug cyclophosphamide. However, methods III and IV are more advantageous concerning the total time of analysis (10 min vs 30 min for methods I and II), they are more sensitive as demonstrated by the lower values of LOD and LOQ (Table 4), in addition, they are environmentally friendly as all the reagents were prepared as aqueous solutions and distilled water was used as the diluting solvent.

4. Conclusion

In this work, four simple sensitive spectrophotometric methods were developed for the determination of MSN in bulk powder and in commercial ampoules. The proposed methods are characterized by being highly sensitive, simple and reliable. Sensitivity was illustrated by the low values of LOD and LOQ. Simplicity was demonstrated by the absence of extraction procedures. Reliability was guaranteed by the results obtained from testing various validation parameters and the successful application to commercial dosage form. Also, the proposed methods are considered to be economic; with low solvent consumption and they could be applied in quality control laboratories where modern equipments are not available. The absence of interference from added excipients is a noted advantage. In addition, reproducibility, accuracy and rapidity of the methods enabled them to be used for the routine determination of MSN in any laboratory.

Conflict of interest

None declared.

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