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Visible Spectrophotometric Determination of Furosemide in Dosage Forms Following its Hydrolysis and Diazo Coupling with Chromotropic Acid

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Several previously reported visible spectrophotometric methods for the quantification of furosemide in dosage forms are fraught with poor specificity due to background absorptivities of other chemical species in the sample matrices.

Objective: To develop a simple, sensitive and specific visible spectrophotometric method for the quantitative determination of furosemide in bulk and dosage forms.

Method: The new spectrophotometric method was based on acid-hydrolysis of furosemide followed by its diazotization and coupling with chromotropic acid to generate a red adduct. Reactions variables critical to optimal response were established. Various analytical and validation parameters including repeatability, reproducibility and selectivity were also determined.

Results: The calibration graph was linear between 8.09 µg/mL to 161.8 µg/mL at 503 nm with a correlation coefficient of 0.992. The Sandell's sensitivity of the new method was 0.14 µg.cm^{-2}/0.001 A.U. while the limits of detection and quantification were 0.75 and 2.28 µg/mL respectively. The method was accurate and precise with recovery in the range of 102.24–109.92% and intra- and inter-day precision (%RSD) at three different concentrations less than 2.0%. When applied to the analysis of dosage form, there was no statistical difference between the newly developed and official methods. There was no interference from commonly used excipients or background absorptivities in the sample matrices.

Conclusion: In comparison with some previously reported colorimetric methods, the new method reliably quantified furosemide over a wider range of concentration and with a superior level of sensitivity. The new method can serve as a reliable alternative to the official method for analysis of furosemide.

Keywords: Furosemide, Colorimetric analysis, Bromotropic acid, Diazo coupling reaction

INTRODUCTION

Furosemide, 4-chloro-*N*-(2-furyl-methyl)-5-sulfamoyl-anthranilic acid, is a powerful loop diuretic that inhibits the absorption of chloride and sodium within the kidney, thereby, increasing the rate of formation of urine and excretion of sodium (Oh and Han, 2015). The drug is as a result used mainly in treating edematous states associated with cardiac chronic renal failure, hypertension, congestive heart failure and cirrhosis of the liver (B.P. 2018; Oh and Han, 2015; Khan et al., 2022). Furosemide is included in the banned drug list of the World anti-doping agency due to the fact that it may mask other drugs (Thevis et al., 2020). Therefore, the accessibility of a valid and simple analytical method for determining the drug in various sample matrices including pharmaceutical dosage forms is very important.

Although the British Pharmacopoeia methods for the quantification of furosemide in bulk and dosage forms

are based on titrimetry and UV spectrophotometry, respectively (B.P. 2018), furosemide has been successfully quantified in both biological and dosage forms using liquid chromatography with UV, fluorescence, or mass spectrometry detection (Galaon et al., 2007; Vree et al., 1994; Youm and Youan, 2013; Zendelovska and Trajce, 2006).

While titrimetry suffers the usual drawbacks of typical classical methods of analysis, liquid chromatographic systems involve the use of sophisticated equipment with high cost of maintenance. Consequently, spectrophotometric methods of analysis because of their inherent simplicity, sensitivity and cost-effectiveness has continued to be the technique of choice in the quality control of furosemide. Previously reported methods generally involved the alkaline or acidic hydrolysis of furosemide to generate a primary amine which is subsequently coupled with chromogenic agents (Ruiz-Angel et al., 2006). Derivatizing agents that have been used in this regard include 3,5-dimethylphenol at 434 nm and resorcinol at 430 nm (Abdullah and Muhammed, 2021; Ali and Muhammed, 2018). Visible spectrophotometric methods based on the oxidation of furosemide with molybdenum (VI) ion or potassium per manganate (Issopoulos, 1989; Kalsang et al., 2009) as well as charge transfer complexation with bromophenol blue or xylenol orange (Saleem et al., 2021) have also been described. Unfortunately, the use of visible spectrophotometric methods in the analysis of furosemide is limited by a general lack of specificity in which overlapping spectra due to other absorbing chemical species are quite common (Ruiz-Angel et al., 2006)

We have previously reported the use of chromotropic acid as an effective derivatizing agent *via* aromatic diazonium coupling (Adegbolagun et al., 2018; Adegoke et al., 2018). Chromotropic acid has been demonstrated to couple rapidly and in a concentration-dependent manner to generate azo adducts with pronounced bathochromic shifts (in excess of 500 nm analytical wavelength) that can permit the quantification of an analyte in the presence of potential interference (M. Abou-Attia et al., 2002).

In this study, we therefore report a simple and cost-effective visible spectrophotometric method based on the diazotization of hydrolyzed furosemide and its subsequent diazo coupling with chromotropic acid.

METHODOLOGY

Materials and reagents

All solvents, salts and acids used in this study are of analytical grade including methanol, (BDH UK) ethanol, (BDH UK), hydrochloric acid (BDH UK), sulphamic acid (Qualikems, India), chromotropic acid (Sigma Aldrich USA), sodium nitrite (Trust Chemical Laboratories), talc, lactose, starch, magnesium stearate. Distilled water was used for all preparations and dilutions.

Instrumentation

UV-Visible spectrophotometer (Spectrumlab 752s) equipped with a 1cm matched quartz cell, Analytical balance (Ohaus UK), magnetic stirrer (Gallenkamp, UK), ultraviolet lamp 254/364nm (PW Allen and Co, London), vortex mixer (Griffin and George, UK), thermostat-controlled water bath (Uniscope)

Preparation of standard solutions

A 1% w/v stock solution of chromotropic acid was prepared by dissolving 0.25 g of chromotropic acid powder in 15 mL of distilled water in a 25 mL volumetric flask and then made up to volume with water. A 3.5M HCl solution was prepared by diluting 5.81 mL of conc. HCl to 20 mL with water in a volumetric flask.

Hydrolysis reaction

20 mg of furosemide (reference standard) was dissolved in 20 mL of methanol before the addition of 3.3 mL 3.5 M HCl and 10 mL water. The mixture was then refluxed at 70˚C for 3 hours after which it was cooled in an ice-bath.

Diazotization reaction

2 mL of 10% w/v sodium nitrite solution was added to an equal volume of hydrolyzed furosemide and then stirred at 0℃ for 30 minutes. The reaction was stopped by addition of 0.2 mL of 0.2 M aqueous sulphamic acid solution.

Derivatization reaction

A 0.5mL aliquot of diazotized furosemide stock solution was transferred into a test tube and 0.5 mL chromotropic acid solution was added and then vortex mixed for 5 minutes. The red adduct formed was incubated at 30℃ for 5 mins. Thereafter, the reaction was terminated by cooling in an ice bath and making

the volume up to 5 mL with methanol. The absorbance readings were taken against a reagent blank.

Spot test

Samples for spot test were prepared by mixing diazotized furosemide stock solution and chromotropic acid solution as described and the colour of the mixture was noted after incubation at 30℃ for 5 to 20 mins. Each determination was carried out in duplicate.

Thin layer chromatography

The TLC analysis of the diazonium, chromotropic acid and adduct solutions were carried out by spotting freshly prepared solutions on pre-coated TLC plates and then developed using ethyl acetate and methanol (8:2) and acetonitrile and methanol (6:4) as mobile phases. Developed plates were visualized under 254 and 365 nm UV light.

Optimization of reaction conditions

The analytical wavelength was selected by overlaying the UV-visible spectra of the diazotized drug, chromotropic acid and the coupling product. Reactions variables such as the concentration of reagents, coupling temperature and time that were critical for the development of maximum colour intensity were also optimized.

A 0.5 mL aliquot of the diazonium solution was added to 0.5 mL of 0.1% w/v chromotropic acid solution in a test tube. The reaction mixture was incubated at 30 °C for 5 mins after which the reaction was stopped by cooling in ice and the volume made up to 10 mL with methanol. The absorbance was taken at 503 nm using methanol as the blank solvent. Each determination was done in duplicates. The entire procedure was repeated using 0.2, 0.3, 0.4, 0.5, 1.0, 2.0 and 3.0% w/v solutions of chromotropic acid.

The effect of the volume of chromotropic acid needed for the derivatization of furosemide was investigated by adding 0, 0.2, 0.25, 0.33, 0.5, 0.67, 0.75, 0.8, and 1 mL of 1% w/v chromotropic acid solution to different test tubes and appropriate volumes of the diazonium solution was added to make 1 mL. The resulting adduct was incubated at 30ºC for 20 minutes after which the reaction was stopped by placing in an ice bath and making the volume up to 10 mL with methanol. Duplicate determinations were carried out with the absorbance read at 503 nm against a reagent blank.

The optimization of the coupling temperature and time was achieved using the steepest ascent method (Karnes and March, 1993) at temperatures of 30, 50, 60, and 70 °C and incubation times of 5 and 20 minutes. Into ten test tubes each containing 0.5 mL of freshly prepared diazotized furosemide solution, 0.5 mL of the chromotropic acid solution was added, mixed by vortex for 10 seconds. At the end of the various reaction times and temperatures, the reaction was stopped by cooling in ice and dilution to 10 mL with methanol. The absorbance of each of the mixtures was taken at 503 nm against a reagent blank. Optimization of coupling time at the selected temperature was done at 0, 5, 10, 15, 20 and 30 minutes.

Stoichiometric ratio determination

Into different test tubes, containing varying amounts (0, 0.25, 0.33, 0.5, 0.67, 0.75 and 1.0 mL) of the diazotized drug solution, appropriate volumes of chromotropic acid was added to make 1 mL. Each reaction mixture was incubated at 30^0C for 20 minutes after which it was cooled in an ice bath before making up the volume to 10 mL with methanol. Absorbance values were taken at 503 nm against a reagent blank. Each determination was carried out in duplicates.

Validation

The calibration line was generated for diazotized furosemide reacted with the coupling agent in the range of 8.09 – 161.80 µg/mL in methanol. This new method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures (ICH, 2011). The accuracy and precision of the method were assessed at three concentrations within the lower, mid and upper portions of the calibration curve using four replicates on the same day (intra-day) and on three consecutive days (inter-day). The accuracy of the method was evaluated as the percentage relative error between the measured and actual concentrations of the drug while the precision was expressed in terms of percentage standard deviation. The limit of detection (LOD) and limit of quantification (LOQ) were also determined.

Method selectivity

The recovery of known amounts (equivalent to 80.9 µg/mL) of the drug when present with commonly utilized pharmaceutical aids, including starch, talc, magnesium stearate, lactose and their mixture were also determined using the proposed method.

Application to assay of dosage form

Two brands of furosemide available in the market were analyzed using the new method. The general procedure involved the hydrolysis and diazotization, as previously described, of an accurately weighed amount of the powdered tablets that is equivalent to 20 mg of furosemide. A 100 µL aliquot of the diazotized solution was then added to 0.67 mL 1%w/v chromotropic acid in a test tube and incubated at 30ºC for 20 minutes. The reaction was stopped by cooling in an ice bath and the volume made up to 10 mL with methanol. The absorbance reading was taken at 503 nm against a reagent blank.

The official method of analysis was also carried out on the dosage forms (B.P. 2018). Six replicate determinations were carried out for each brand using both methods.

RESULTS AND DISCUSSION

Evidence of coupling

Diazo coupling yielded a red adduct at both 5 and 20-minute incubation times. The adduct was stable for up to 24 hours when monitored by absorbance values. The TLC analysis in the different mobile phases revealed the formation of a single-component adduct with Rf values that were distinct from those of diazotized furosemide and the coupling agent.

Selection of analytical wavelength

The overlaid spectra of diazotized furosemide, chromotropic acid and adduct is shown in Fig. 1. The analytical wavelength was selected as 503 nm as it showed the largest difference between the absorptivities of the adduct and the other reagents. The UV spectrum of the adduct showed large bathochromic shift and hyperchromic change with respect to the diazonium or derivatizing agent. The large hyperchromic and bathochromic changes herein reported thus resolved a well-known problem of lack of specificity (as a result of overlapping spectra) which characterized several previously reported methods of quantification for furosemide (Abdullah and Muhammed, 2021; Ali and Muhammed, 2018; Dill Rani and Venkateswarlu, 2017; Ruiz-Angel et al., 2006).

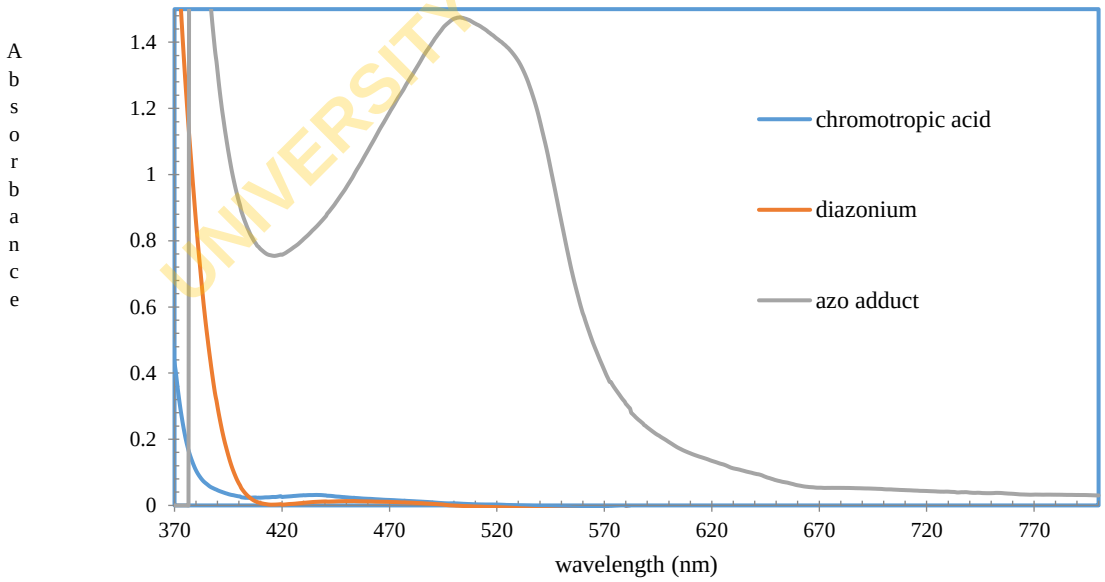


Figure 1: Overlaid spectra of diazotized furosemide, chromotropic acid and adduct

Selection of reagent concentration

Appropriate reagent concentrations for the study was selected by measuring the absorbance value of different concentrations of chromotropic acid at 503 nm. The concentration (1% w/v) that gave the highest absorbance was selected for the study. This is presented in Figure 2.

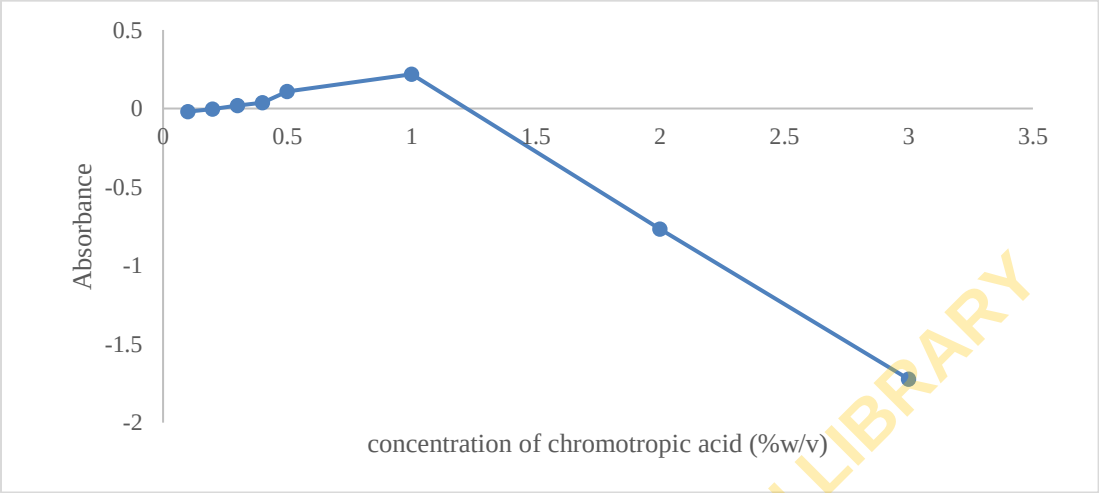


Figure 2: Variation in absorbance values with concentration of derivatizing agent

Selection of coupling temperature and time

For both incubation times of 5 and 20 minutes, the absorbance gradually increased with temperature and then plateaued at 50 ℃. However, as shown in Figure 3, the difference in absorbance values at 30 and 50℃ were not sufficiently different to justify working at elevated temperatures. The optimum coupling temperature was therefore established at 30 ℃. Optimization of coupling time at the selected temperature revealed maximum response at 20 minutes.

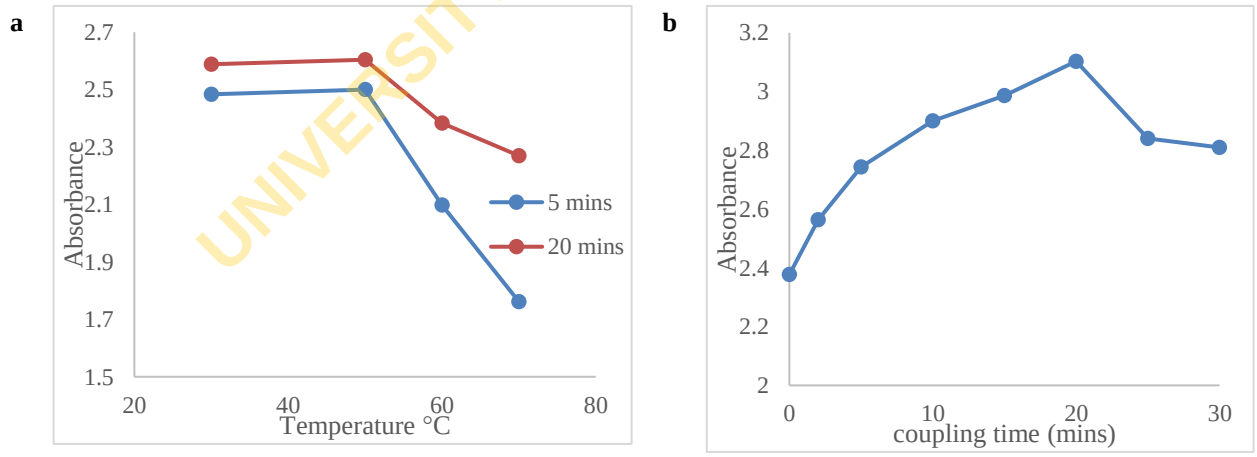


Figure 3: Optimization of coupling (a) temperature and (b) time for the formation of azo adduct

Stoichiometric ratio determination and reaction mechanism

Optimal response was observed when the diazotized furosemide combined with chromotropic acid in 1:2 ratio as shown in Fig. 4. Although this result slightly differs from the 1:1 stoichiometric ratio obtained in our previous studies with chromotropic acid (Adegbolagun et al., 2018; Adegoke et al., 2019), there exists sufficient proof that furosemide hydrolyses in hydrochloric acid to yield an anthranilic derivative with

a free amino acid group (Cruz et al., 1979). The primary aromatic amine can therefore be converted to the diazonium salt in the presence of nitrous acid and subsequently react with the activated ring system of chromotropic acid to generate the coloured azo adduct in a 1:1 ratio (Scheme 1). The formation of a single-product as obtained in the TLC analysis also confirmed this mechanism.

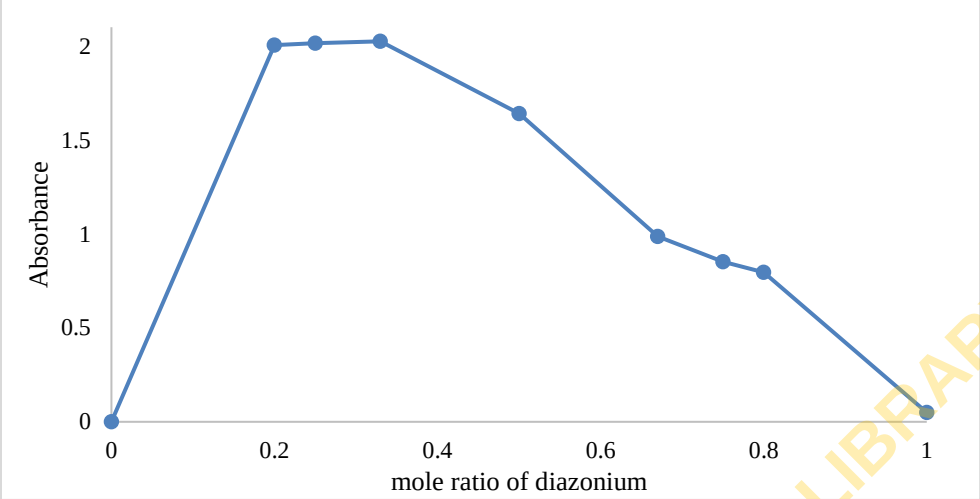
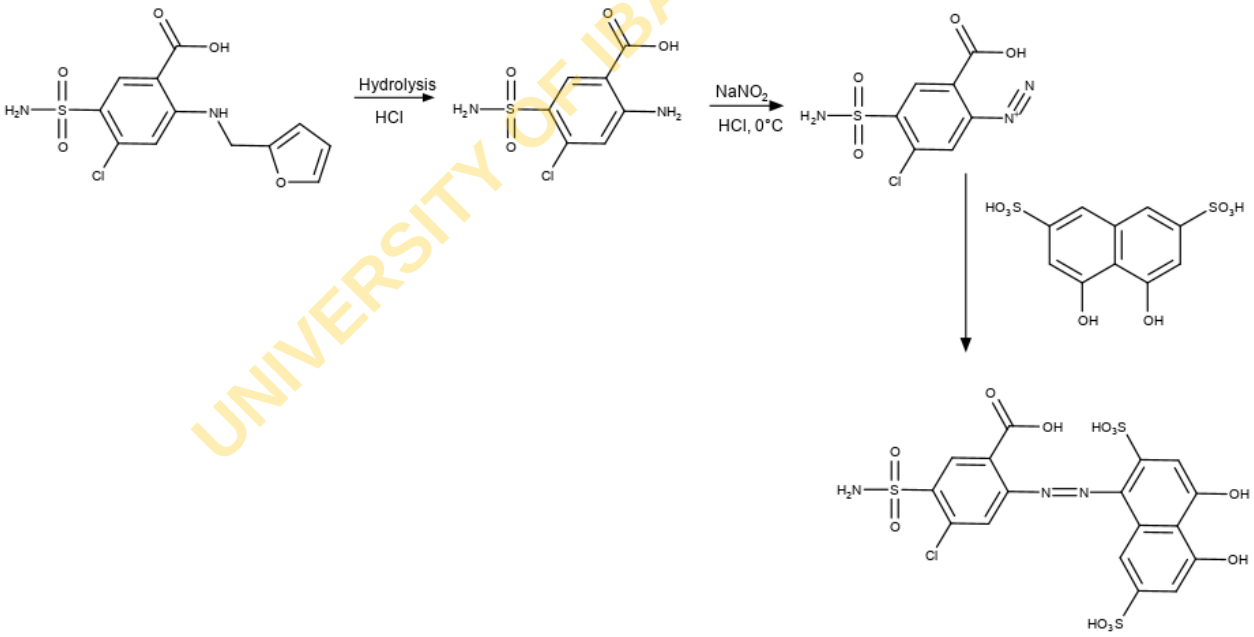


Figure 4: Variation of absorbance with mole ratio of diazonium solution



Scheme 1: Proposed mechanism for the reaction between diazotized furosemide and chromotropic acid.

Validation of the proposed method

The linearity of an analytical method is its ability to elicit response parameters that are directly (or following mathematical transformation) proportional to the concentration of the analyte. Linearity of a method is often defined in terms of the extent of variance around the slope of a regression equation derived from the Beer-Lambert relationship between absorbance and increasing concentrations of the

analyte. A linear relationship was observed between absorbance at 503 nm and concentration of furosemide in the range of 8.09-161.80 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 3.3σ/S and 10σ/S, respectively where, σ is the standard deviation of the absorbance of blank (n = 6) and S is the slope of the calibration line (ICH, 2011). The various analytical parameters are presented in Table 1.

Table 1. Analytical and validation parameters for proposed method

Performance parameter	Value
Beer's law limit (µg/mL)	8.09-161.80
Limit of detection (µg/mL)	0.75
Limit of quantification (µg/mL)	2.28
Molar absorptivity ($Lmol^{-1}cm^{-1}$)	$2.32x10^3$
Sandell's sensitivity (µg mL^{-1} per 0.001 absorbance unit)	0.14
Slope	0.007
Intercept	0.2016
Coefficient of determination	0.992
Interference liabilities (% furosemide)	98.5±0.004 - 99.5±0.01
Standard deviation of intercept	0.001

The accuracy and precision of the new method were carried out over a three-day period as depicted in Table 2. Accuracy is a measure of the agreement between the measured value obtained with a method and the true value. The accuracy of the newly reported method was expressed as mean recoveries while its precision was expressed in terms of variation in the relative standard deviation. The intra-day results revealed a mean recovery of 102.24-109.44 % when the new method was applied to replicate sample matrices spiked with known amounts of the analyte. The method also showed good repeatability as the percentage RSD did not exceed 1.32 %.

For the inter-day results, the percentage recovery of the new method was 102.65-109.92 %. The percentage relative standard deviation was also less than 2.0 % indicating good reproducibility. Very similar reproducibility were obtained with previously reported colorimetric methods based on derivatization with resorcinol, 3,5-dimethylphenol or 2,3 dichloro-5,6-dicyano 1,4-benzoquinone (Abdullah and Muhammed, 2021; Ali and Muhammed, 2018; Dill Rani and Venkateswarlu, 2017)

Table 2. Accuracy and precision of new method

Concentration (µg/mL)	Day 1[a]		Day 2[a]		Day 3[a]		Inter-day statistics[b]	
	Mean recovery (%)	RSD	Mean recovery (%)	RSD	Mean recovery (%)	RSD	Mean recovery (%)	RSD
35.59	109.44±0.51	1.32	107.99±0.29	0.76	109.92±0.51	1.19	109.92±0.51	1.44
64.72	107.08±0.53	0.79	106.73±0.55	0.79	107.35±0.55	0.52	107.35±0.56	0.85
97.08	102.47±0.87	0.87	102.24±0.86	0.87	102.65±0.86	0.43	102.65±0.86	0.89

[a]n=4; [b]n=12

Method selectivity

The ability of the new method to selectively quantify furosemide in the presence of commonly utilized pharmaceutical excipients was established. The results showed that the method has a recovery between 98.5 to 100.4% when applied to spiked amounts of furosemide in sample matrices variously containing starch, talc, magnesium stearate, lactose and their mixture.

Application to dosage form analysis

The proposed method was applied to two brands of furosemide with label claims of 40 mg active ingredient. The results obtained were statistically compared with those obtained using the official method (B.P. 2018) as shown in Table 3. The mean recovery determined as the ratio of recovery obtained with new method to the official method was 100.50-101.95%. The percentage error of the new method, expressed as the difference between the measured and reference values, did not exceed 2.21%. The Student's t test was used to compare the mean recoveries of the new and official methods, with 95% confidence intervals.

Table 3. Comparative dosage form analysis with new and official methods

Drug formulation	New method		B.P. 2018 method		*Mean recovery±SD(%)	%error	Statistics (p values)
	Amount found(µg/mL)	%RSD	Amount found(µg/mL)	%RSD			T test
Brand 1	84.97	2.84	83.13	0.24	101.95±2.42	2.21	0.16
Brand 2	101.00	1.36	100.49	0.35	100.50±1.37	0.51	0.46

** ratio of the results obtained from the new method to that of the official method*

No significant differences was found in the accuracy and precision of the two methods. The proposed method can therefore serve as a reliable alternative in the routine analysis of furosemide in bulk and dosage form.

Comparison of the new method with previous spectrophotometric methods

A cursory examination of the validation parameters of the newly developed method revealed its superior performance when compared with a number of previously reported visible spectrophotometric methods (Table 4). In comparison, the new method reliably quantified furosemide over a wider range of concentration and with a higher level of sensitivity as indicated by the Sandell's sensitivity. More importantly, the very low-energy analytical wavelength of 503 nm improved the specificity of the new method as the large bathochromic shift precludes the problem of overlapping spectra and background absorptivities of other chemical species in the sample matrices. In addition, the new method does not require an extraction procedure or the strict control of pH with buffers as reported with similarly performing copper chloride, bromophenol and Chromazurol methods (Gölcü, 2006; Saleem et al., 2021).

Table 4. Comparison of the new method with previous spectrophotometric methods

Derivatizing agent	Beer-Lambert limits (µg/mL)	Analytical wavelength (nm)	LOD (µg/mL)	Max. RSD%	Sandell's sensitivity	Reference
Resorcinol	0.25-2.5	430	0.019	1.49	0.007	(Abdullah and Muhammed, 2021)
3,5-dimethylphenol	0.4-50	434	0.127	0.021	0.024	(Ali and Muhammed, 2018)
2,3 dichloro-5,6-dicyano 1,4-benzoquinone	20-160	450	0.51	0.32	0.002	(Dill-Rani and Venkateswarlu, 2017)
Copper II chloride	5-30	790	0.23	1.0	Not reported	(Gölcü, 2006)
Bromophenol	0.4-32	591	Not reported	0.53	0.024	(Saleem et al., 2021)
Chromazurol S	0.8-32	525	Not reported	2.29	0.021	(Saleem et al., 2021)
Chromotropic acid	8.09-161.80	503	0.75	1.44	0.143	This study

CONCLUSION

A novel visible spectrophotometric method for the analysis of furosemide has been developed and validated. The method can serve as a reliable and affordable assay method for the routine analysis of furosemide in bulk and dosage forms.

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