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Development and validation of novel stability indicating methods for estimation of amylocaine hydrochloride in bulk and dosage form

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ABSTRACT

Three sensitive and selective methods were developed and validated as stability indicating methods for the determination of amylocaine HCl in presence of its degradation product. The first method is based on the use of derivative ratio spectrophotometry (^{1}DD) for the determination of amylocaine HCl in presence of its degradation product and of methyl and propyl parabens by measurement of the peak amplitudes of the first derivative of ratio spectra at 234 and 247 nm using the spectrum 20 µg of total parabens as a divisor. The second method is a spectro-densitometric method for the determination of amylocaine HCl after separation from its degradation product and additives of pharmaceutical dosage form using toluene: methanol: chloroform: 10% NH_3 (5:3:6:0.1 v/v) followed by detection at 234 nm. The third method is an isocratic high performance liquid chromatographic method (HPLC) on a reversed phase C_{18} column using mobile phase consisting of distilled water: acetonitrile: triethylamine (530: 470: 0.1v/v) and the pH was adjusted to 3 by *o*-phosphoric acid. The proposed methods were successfully applied for the analysis of amylocaine HCl in laboratory prepared mixtures and in pharmaceutical dosage form and the results obtained were assessed by applying the standard addition technique. Statistical comparison between the results obtained by applying the proposed methods and manufacturer's method for amylocaine HCl in its pure powder form was done and no significant difference was found at p = 0.05.

KEYWORDS

Derivative ratio spectrophotometry;
Spectro-densitometry;
High performance liquid chromatography;
Amylocaine HCl;
Benzoic acid.

INTRODUCTION

Amylocaine HCl (AM), (1-(Dimethylaminomethyl)-2-methylbutan-2-yl) benzoate hydrochloride (Figure 1) is a benzoic acid ester local anaesthetic used mainly for relief of painful anorectal conditions and has been included in oral mixtures for the relief of coughs. It blocks the initiation and conduction of nerve impulse by inhibiting

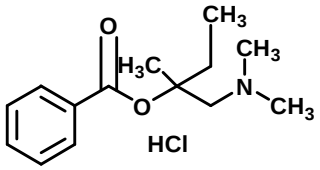

Figure 1: Chemical structure of amylocaine HCl.

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permeability of the cell membrane to Na^+ thus prevents depolarization of nerve membrane[1]. Several methods have been described for the determination of AM. These include HPLC[2-5], TLC[6], capillary zone electrophoresis[7], atomic absorption spectrometry[8-10] and electrochemical methods[11]. According to literature in our hand there is no any stability study of amylocaine therefore, the aim of this work is to develop simple, accurate and precise stability indicating methods for the determination of AM in presence of its degradation product in addition to the additives of pharmaceutical dosage form.

EXPERIMENTAL

Instruments

UNICAM UV 300, Spectrophotometer, thermospectronic with vision32 software, connected to IBMPC computer and HP laser jet 1100 series printer was used. The absorbance of all samples was recorded against a solvent blank in 1 cm quartz cuvettes and saved in the computer by scanning at band width 1.5 nm, scan speed 1200 nm min^{-1}, (England).

TLC aluminum plates (20 cm × 20 cm (with 0.2 mm silica Gel $60F_{254}$ were purchased from Merck (Germany) and the samples were applied to the plates with a 10 µl Chromatopak micro syringe (India). A Shimadzu dual wavelength flying spot densitometer, Model CS-9301 PC (Tokyo – Japan) was used. The measurements conditions were as follows: wavelength = 235 nm; photo mode = reflection; scan mode = zigzag; swing width = 10.

HPLC system, Agilent technologies 1100 series consists of Agilent 1100 -series Variable Wavelength Detector, Agilent 1100 - series Vacuum degasser, Agilent 1100 - series Manual injector and Agilent 1100 - series Quaternary pump G1311A. The column is Novapak C_{18} 5µm, 25 cm × 4.6 mm HPLC Cartridge Column (USA). Agilent Syringe, LC100 µl, (USA) was used. Cellulose acetate filter papers, dimension 47 mm, pore size 0.45 µm, Rankem, (New Delhi) were used.

Samples

Standard samples

Amylocaine HCl was kindly supplied by Alexandria Co. for pharmaceuticals, Alexandria, Egypt and its purity was found to be 100.07±1.251 according to the manufacturer's method. Propyl paraben and methyl paraben were kindly supplied by Mash Co. for Pharmaceutical Industries, Badr city, Egypt and their purities were labelled to be 100.40% and 98.55% respectively. Pepsin, papin and diastase were kindly supplied by Novartis Pharma, Cairo, Egypt. Benzoic acid was purchased from Sigma Aldrich - USA.

Commercial product

Postine-S syrup® (batch no. 410640) labeled to contain 0.05 gm/100ml AM, 0.24 gm/100ml Pepsin, 1.662 gm/100ml papin, 0.1 gm/100ml diastase, 0.06 gm/100ml methyl paraben and 0.04 gm/100ml propyl paraben manufactured by Alexandria Co. for pharmaceuticals, Alexandria, Egypt.

Chemicals and solvents

The water for HPLC was prepared by double glass distillation and filtration through a 0.45 µm membrane filter (Rankem, New Delhi). Methanol, ortho-phosphoric acid, triethylamine and acetonitrile used were of a HPLC grade (Lab. Scan, Ireland), other solvents chloroform, hydrochloric acid (Fischer Scientific, UK), toluene, ammonia, and potassium hydroxide (Al-Nasr Pharmaceutical Chemical Co., Cairo, Egypt) were of an analytical grade.

Solutions

- Amylocaine HCl stock standard solution 1mg/ml in methanol.
- Amylocaine HCl working solution 0.4 mg/ml in methanol (for ^{1}DD).
- Amylocaine HCl working solution 0.04 mg/ml in methanol (for HPLC).
- Amylocaine HCl working solutions 0.5- 5.5 mg/ml (for TLC).
- Benzoic acid stock solution 1mg/ml in methanol.
- Benzoic acid working solution 0.4 mg/ml in methanol (for ^{1}DD).
- Benzoic acid working solution 0.04 mg/ml in methanol (for HPLC).
- Total parabens stock solution 1mg/ml in methanol.
- Total parabens working solution 0.1mg/ml in methanol. (3: 2 of methyl paraben: propyl paraben, respectively).

Prparation of induced degradation-product

An accurately weighed amount about 50 mg of powdered AM was refluxed with 50 ml 2N hydrochloric acid or 2N NaOH for 4 hours, and then the solution was neutralized with 2N sodium hydroxide or 2N HCl. The solution was concentrated to about 5 ml and quantitatively transferred to 50-ml volumetric flasks. The volume was completed with methanol. Complete degradation was checked by using TLC, using toluene-methanol-chloroform-10% ammonia (5: 3: 6: 0.1 v/v) as developing solvent.

Procedures

Chromatographic conditions

(a) TLC-densitometry

TLC was performed on 20 cm X 20 cm TLC aluminum sheets precoated with 0.2 mm silica Gel 60 F_{254}, the plates were prewashed with methanol and activated at 100°C for 5 min. The samples were applied as compact spots 15 mm apart and 15 mm from the bottom of the TLC plate using 10 µl micro syringe. Linear ascending development was done in a chromatographic tank previously saturated with toluene-methanol-chloroform-10% ammonia (5: 3: 6: 0.1 v/v) for 30 minutes at room temperature to a distance of approximately 15 cm from the lower edge. The developed plates were air dried and scanned at 235 nm on Shimadzu dual wavelength flying spot densitometer operated in photo mode = reflection; scan mode = zigzag; swing width = 10.

(b) HPLC method

HPLC was carried out at ambient temperature on RP C_{18} column (5µm, 25 cm x 4.6 mm, Novapak). The mobile phase consisted of distilled water: acetonitrile: triethylamine (530: 470: 0.1v/v) and the pH was adjusted to 3 by *o*-phosphoric acid. The mobile phase was filtered using 0.45 µm membrane filter (Rankem, New Delhi) followed by degassing with ultrasonic vibration prior to use and was delivered at 1ml/min. The injection volume was 20 µl. The column was conditioned for at least 30 min. The detection was achieved with UV detection at 235 nm.

Linearity

(a) Spectrophotometric method

The recorded absorption spectra of AM in concentration range 20-140µg/ml which prepared by appropriate dilution from working solution of AM (40µg/ml) were divided by spectrum of 20 µg of total parapens and the first derivative of the ratio spectra curves was obtained. The calibration curve was plotted relating AM concentration to the corresponding peak amplitudes of the first derivative of ratio spectra curves at 234 and 247 nm.

(b) TLC method

Triplicate 10µl aliquots were separated applied to TLC plates from working standard solution of AM (0.5-5.5 mg/ml). The plates were chromatographed and scanned as under chromatographic conditions. The calibration curve relating the integrated area under the peak to the corresponding concentration (µg/spot) was constructed.

(c) RP-HPLC method

Appropriate dilution from working solution of AM (40µg/ml) was done to prepare solutions of concentration ranging from 2-14 µg/ml. Triplicate 20 µl samples were injected and chromatographed as under chromatographic conditions. The average peak areas were calculated and plotted against the corresponding concentrations.

Specificity

The proposed procedures were applied for the analysis of laboratory prepared mixtures containing different ratios of degradation product and the content of AM was determined from the corresponding regression equations.

Analysis of pharmaceutical dosage forms

A sample of 50-ml of drug product solution was transferred into a 250-ml separating funnel followed by addition of 20 ml 33% ammonia solution to liberate Am free base which was extracted with 100 ml ether. The aqueous layer was extracted three times with 20ml of ether each then the combined ether extract was washed with 20 ml 10% ammonium hydroxide solution three times till no pink color is present. The ether extract was evaporated and the residue was dissolved in 5 ml 0.1N HCl then the volume was completed to 25ml using methanol to produce stock solution of 1 mg/ml. Further dilutions of the stock solution were made with methanol for (^{1}DD and TLC methods) or

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with mobile phase for (HPLC method). The concentrations of Am were calculated from the corresponding regression equations.

Application of standard addition technique

The standard addition technique was carried out by adding different known concentrations of pure AM to a known concentration of the commercial dosage form then proceeding as described in the mentioned methods.

RESULTS AND DISCUSSION

By reviewing literature in our hand we found that there is no stability-indicating method has been reported for the determination of AM in the presence of degradation product. Since the international Conference on Harmonization (ICH) guideline entitled 'stability testing of new drug substances and products[12] requires that stress testing should be carried out to elucidate the inherent stability characteristics of the active substance. Susceptibility to hydrolysis is one of the required tests, therefore the aim of this work was to study the stability of AM and to develop stability indicating methods for its determination. AM was subjected to acid and alkaline hydrolysis and the degradation process was followed by TLC separation using toluene-methanol-chloroform-10% ammonia (5: 3: 6: 0.1 v/v) as a developing system. It was found that complete degradation occurred after 4 hours reflux with 2N HCl or 2N NaOH as indicated by disappearance of the spot corresponding to AM and appearance of another spot at R_f = 0.29 corresponding to benzoic acid. The difficulty of developing a stability indicating method for the determination of AM was increased by the presence of methyl and propyl parabens in dosage form. The zero spectra of total parabens and benzoic acid overlap with the spectrum of AM that hinders its direct determination (Figure 2). Simple derivative spectrophotometric techniques (first to fourth) were investigated but they failed to solve this overlap. Therefore, first derivative of ratio spectra spectrophotometric method (^{1}DD) was investigated to solve this problem. The influence of the variables such as, divisor concentration and smoothing function were optimized. A correct choice of the divisor concentration is fundamental. Bad choice of the divisor concentration may lead to great noise of ratio spectra. If the concentration of divisor is increased or decreased, the resulting derivative ratio values are proportionally decreased or increased with consequent variation of both sensitivity and linearity range. From several trials the best results in terms of signal to noise ratio, accuracy, sensitivity and repeatability were obtained upon using 20 µg/ml of total parabens as a divisor. The concentration of AM was found to be proportional to the peak amplitude of the first derivative ratio ^{1}DD curves at 234 and 247nm in the concentration range 2-14 µg/ml (Figure 3). The regression equations were also computed (TABLE 1).

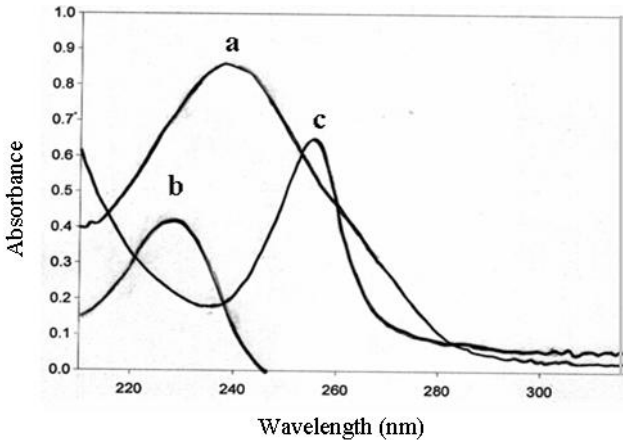

Figure 2 : Zero order absorbtion spectra of (a) 100 µg/ml amylocaine HCl, (b) 100 µg/ml benzoic acid and (c) 20 µg/ml of total parabens

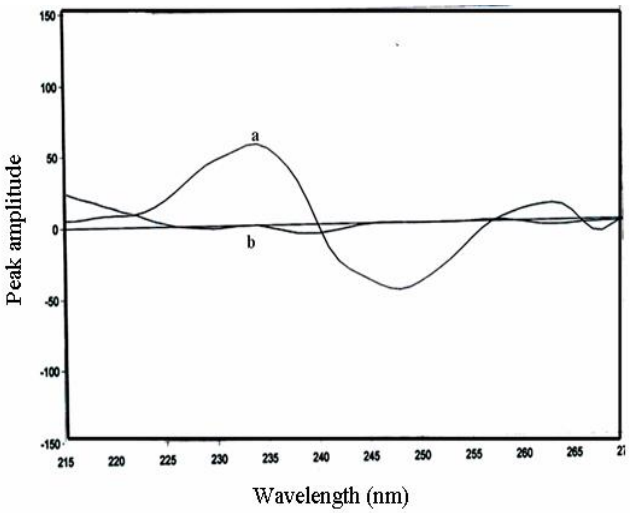

Figure 3 : First derivative of ratio spectra of (a) 100 µg /ml amylocaine HCl, (b) 100 µg /ml benzoic acid using 20 µg /ml of total parabens as divisor.

Two sensitive stability indicating chromatographic methods were also suggested for the determination of

Am in the presence of degradation product and parabens. For the TLC method the experimental conditions such as mobile phase composition, scan mode and wavelength of detection, were optimized to provide accurate, precise and reproducible results. The best results were obtained using toluene-methanol-chloroform-10% ammonia (5: 3: 6: 0.1 v/v) as developing solvent, scan mode was the zigzag mode and the wavelength of scanning was chosen to be 235 nm. Under this conditions, AM was separated from its acid degradant and total parabens (R_f values 0.62, 0.29 and 0.42 for AM, acid degradant, total parabens, respectively) Figure 4. Linear relationship was found to exist between the integrated peak area of the separated spots and the corresponding concentration of AM and the regression equation was also computed (TABLE 1). For the HPLC method, the assay parameters were optimized. A satisfactory separation was obtained with a mobile phase consisting of distilled water: acetonitrile: triethylamine (530: 470: 0.1v/v) and the pH was adjusted to 3 by *o*-phosphoric acid Figure 5. Increasing acetonitrile concentration (by 10 %) lead to inadequate separation of AM and its degradant. At lower acetonitrile concentration (by 10 %) separation occurred but with excessive tailing and increased retention time for AM peak. At lower or higher apparent pH value (± 2 pH units), bad resolution was observed for AM and its degradant. Quantitation was achieved with UV detection at 235 nm based on measuring area under the peak. The system suitability parameters of HPLC method were calculated according to USP[13] and the results showed good resolution of the Am and benzoic acid, TABLE 2. Where parabens concentration presented in dosage form is lower than that can be detected under the proposed HPLC chromatographic condition so the chromatogram does not show any peaks corresponding to papabens. The average retention time ± SD for AM and its degradant were found to be 1.363 ± 0.0025 min 2.257 ± 0.0024 min, respectively. A linear relationship was obtained between the integrated peak area at the selected wavelength (235 nm) and the corresponding concentrations in the range of 0.2-1.4 µg/ml AM (TABLE 1).

TABLE 1 : Validation and regression equations parameters for the proposed methods for the determination of amylocaine HCl in pure powder form.

Parameter	Spectrophotometry		TLC	HPLC
	at 234 nm	at 247 nm		
LOD	0.30 µg /ml	0.50 µg /ml	2.5 µg /spot	0.50 µg /ml
LOQ	20.0 µg /ml	20.0 µg /ml	20 µg /spot	2.0 µg /ml
Linearity range	20.0 -140.0 µg/ml	20.0 - 140.0 µg/ml	5.0 - 55.0 µg/spot	2.0-14.0 µg/ml
Accuracy				
-Mean	99.62	99.554	100.13	99.59
-R.S.D.	1.574	0.822	1.323	1.765
Precision				
Result 1	1.125	0.774	1.009	1.036
Result 2	0.963	1.258	0.205	0.698
Result 3	1.045	0.426	1.200	0.766
Regression Eq.				
Slope	0.4828	0.3859	144.667	397.1225
S.E. of Slope	0.0431	0.049	20.245	55.823
CL[a] of slope	0.4717 – 0.4939	0.3732 – 0.3985	139.055 -150.297	382.772 -411.472
Intercept	-0.778	- 0.193	635.27	8.004
S.E. of intercept	0.385	0.439	69.88	49.930
CL[a] of intercept	-1.77 – 0.213	-1.323 -0.937	441.229 – 829.309	-120.34-136.35
correlation coefficient	0.9997	0.9995	0.9992	0.9994

[a] **CL the confidence limit.**

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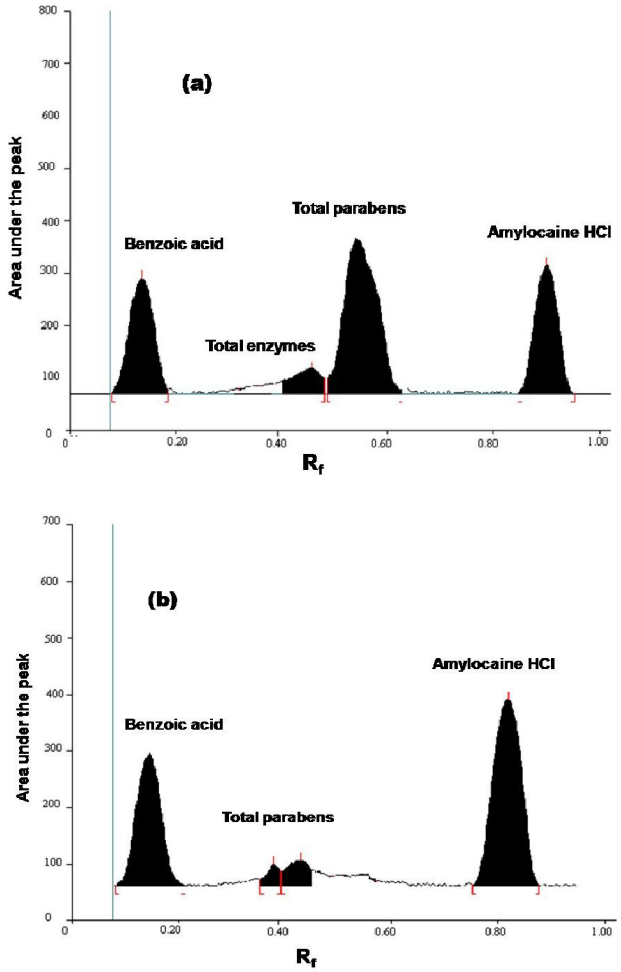

Figure 4: Thin layer chromatograms of (a) Laboratory mixture containing Am, benzoic acid, total parabens and total enzymes. (b) Extract of dosage form, using toluene- methanol- chloroform 10% aqueous ammonia (5: 3: 6: 0.1, *v/v*) as developing system, at ambient temperature (25°C)

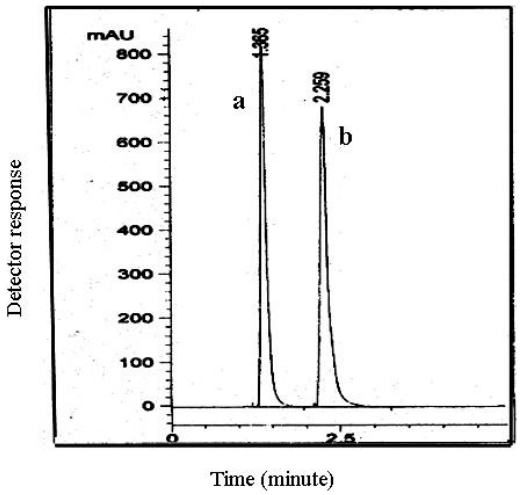

Figure 5: HPLC chromatogram of 20 µl of mixture containing of postine-S equivalent to 10 µg /ml of amylocaine HCl (a) and 10 µg / ml of benzoic acid (b)

TABLE 2 : Parameters required for the system suitability test of HPLC method.

Parameter	Obtained values		Reference values
	amylocaine	Benzoic acid	
Resolution	8.47		R > 1.5
T (tailing factor)	1.22	1.35	T = 1 for a typical symmetric peak
α (relative retention time)	2.03		> 1
K (column capacity)	1.72	3.5	1-10 acceptable
N (column efficiency)	1458	2473	Increases with efficiency of the separation
HETP = L (length of column in cm)/N	0.017	0.01	The smaller the value, the higher the column efficiency

Method validation

ICH guidelines for the method validation[14] were followed for validation of the suggested method.

Linearity and range

Under the previously described experimental condition linear relationships were obtained by plotting the drug concentration against peak amplitude or peak area for spectrophotometric or chromatographic methods, respectively. The corresponding concentration ranges, calibration equation, LOD, LOQ and other statistical parameters are listed in TABLE 1.

Accuracy

The accuracy of the investigated methods was validated by analyzing pure samples of AM in triplicate. The concentrations of the active drugs were calculated from the corresponding regression equations. Good results were obtained as shown in TABLE 1.

Precision

Precision was evaluated by calculating intra- and inter-day precision after repeating the assay of three different concentrations three times in the same day and assaying the samples in triplicate on three successive days using the proposed methods. The calculated RSD were listed in TABLE 1 indicating satisfactory precision of the proposed methods.

Specificity

The specificity of the proposed methods was checked by analyzing different synthetic mixtures containing different ratios of AM and its degradation

product by the proposed methods. Satisfactory results were obtained and shown in TABLE 3.

TABLE 3 : The determination of AM in laboratory prepared mixtures containing different ratios of AM and benzoic acid by the proposed spectrophotometric and chromatographic methods.

Spectrophotometric method					TLC				HPLC		
Concentration (µg/ml)			Recovery % at 234	Recovery % at 247	Concentration (µg/spot)			Recovery %	Concentration (µg/ml)		Recovery %
AM	Benzoic acid	Total parabens[a]			AM	Benzoic acid	Total parabens[a]		AM	Benzoic acid	
20	100	20	101.91	102.09	5	10	40	98.62	2	12	99.54
40	96	4	100.99	101.61	10	15	30	98.09	4	10	98.17
60	70	10	102.00	101.37	20	25	10	100.97	6	8	100.56
80	44	16	100.54	99.81	30	5	20	99.64	8	6	98.91
100	35	5	99.98	99.64	40	210	5	98.54	10	4	100.54
120	6	14	101.65	102.11	50	2	3	98.73	12	2	101.64
Mean ± R.S.D.			101.18 ±0.804	101.11± 1.095				99.10 ±1.057			99.89 ± 1.264

[a] Ratio of methyl to propyl parabens.

Application of commercial dosage form

The proposed methods were successfully applied for the determination of AM in commercial syrup. The results shown in the TABLES 4 and 5 were satisfactory with good agreement with the labeled amount. Moreover, to check the validity of the proposed methods, the standard addition technique was applied. The recovery of AM was calculated by comparing the concentration obtained from the spiked mixtures with those of the pure AM. The results obtained (TABLES 4 and 5) suggested that there is no interference from excipients, which are normally present in syrup.

TABLE 4: The results of analysis of AM in Postine-S syrup®[a] and application of the standard addition technique by the proposed spectrophotometric method.

Postine-S syrup®[a] Recovery % ± R.S.D		Claimed amount Taken (µg/ml)	Stand. added (µg/ml)	Recovery %	
234 nm	247 nm			234 nm	247 nm
98.66 ± 0.714	100.23 ± 1.508	20	20	101.36	101.37
			60	101.17	102.14
			80	101.84	101.05
			100	102.10	100.69
Mean ± RSD%				101.62 ± 0.421	101.56 ± 0.458

[a] Batch No. 410640

TABLE 5 : The results of analysis of Postine-S syrup®[a] and application of the standard addition technique to the analysis of AM in Postine-S syrup®[a] by the proposed chromatographic methods.

TLC				HPLC			
Recovery % ± R.S.D	Claimed amount taken (µg/spot)	Stand. added (µg/spot)	Recovery %	Postine-S syrup®[a] batch no. 410640 Recovery % ± R.S.D	Claimed amount taken (µg/ml)	Stand. Added (µg/ml)	Recovery %
99.46 ± 1.175	5.0	5.0	100.69	99.48 ± 1.340	2.0	2.0	100.54
		10.0	101.63			4.0	99.16
		30.0	100.12			8.0	99.09
		50.0	99.87			10.0	98.98
Mean ± RSD %			100.57 ± 0.776				99.44 ± 0.739

[a] Batch No. 410640

Robustness

Robustness of the methods was assessed by evaluating the influence of small change of experimental conditions, such as composition of mobile phase or developing system, pH and flow rate, and the chromatographic resolution was evaluated. The results indicate that the method remains unaffected by small changes in conditions. This provided an indication for the reliability of the proposed methods during routine work.

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Stability

The solutions were found to be stable for 14 days, when stored in tightly capped volumetric flasks, protected from light on a laboratory bench and in refrigerator.

The results of determination of AM in pure powder form obtained from the proposed 1DD, TLC and HPLC methods were compared with those of the manufacturer method. Statistical comparison of the results was performed with regard to accuracy and precision using Student's t-test and the F-ratio at 95% confidence level and no significant difference was found (TABLE 6)

TABLE 6 : Statistical analysis of the results obtained by the proposed methods and the manufacturer's method for the analysis of AM in pure powder form.

Parameters	Spectrophotometric method		TLC	HPLC	Manufacture's method[Personal Communication]
	234 nm	247 nm			
Mean	99.62	99.55	100.13	99.59	100.07
S.D.	1.568	0.818	1.322	1.758	1.251
N	5	5	5	5	5
V	2.458	0.669	1.747	3.090	1.565
Student's t test	0.502 (2.306)[a]	0.778 (2.306)[a]	0.074 (2.306)[a]	0.498 (2.306)[a]	
F test	1.57 (6.39)[a]	2.34 (6.39)[a]	1.12 (6.39)[a]	1.97 (6.39)[a]	

[a] **The values between parenthesis are the theoretical values of t and F at (P = 0.05).**

CONCLUSION

The proposed [1]DD, TLC, and HPLC methods provide simple, accurate and reproducible quantitative analysis for the determination of amylocaine HCl in pharmaceutical product and in the presence of its degradation product. The HPLC method was found to be more specific and selective than the derivative spectrophotometric methods. While the derivative spectrophotometric method has the advantages of lower cost, rapid and environment protecting. The TLC method is simple and uses a minimal volume of solvents, compared to the HPLC method. The proposed methods complied with ICH guidelines.

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