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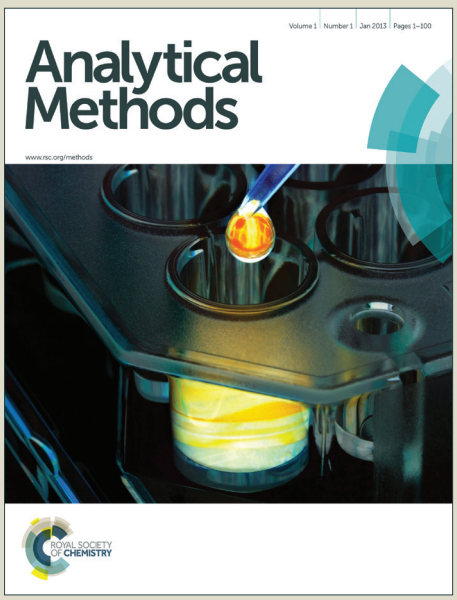

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Validated simultaneous determination of antipyrine and benzocaine HCl in the presence of benzocaine HCl degradation product

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Abstract

Validated, sensitive and highly selective two stability indicating methods are adopted for simultaneous quantitative determination of antipyrine (ANT) and benzocaine HCl (BEN) in the presence of benzocaine HCl degradation product [p-aminobenzoic acid (PABA)]. The first method is high performance liquid chromatography, where the mixture of antipyrine (ANT), benzocaine HCl (BEN) and benzocaine HCl degradation product (PABA), the reported benzocaine degradation product, are separated on C_8 ZORBAX, (5µm, 4.6 x 150 mm I.D.) analytical column using acetonitrile: phosphate buffer pH 5.5 (25: 75, v/v) as a mobile phase. The drugs were detected at 270 nm over a concentration range of 10-100 µg $mL^{-1}$and 5-100 µg mL^{-1} with mean percentage recoveries 100.22% (S.D. 1.375) and 99.77% (S.D. 1.089) for antipyrine and bezocaine HCl, respectively. The second method is Thin Layer Chromatography combined with densitometric determination of the separated bands at 275nm. Adequate separation was achieved using silica gel60 TLC F_{254} plates and toluene: acetone: methanol: ammonia (8:3:3:0.1 by volume) as mobile phase. The proposed methods were applied for the analysis of antipyrine and bezocaine HCl in their pharmaceutical formulation and the results were statistically compared with the reported methods.

Key words: Antipyrine, Bezocaine HCl, HPLC, TLC-densitometry, Stability-indicating method

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1. Introduction

Antipyrine (ANT) is 1, 5-Dimethyl-2-phenyl-4-pyrazolin-3-one, it is a NSAID that can relieve mild to moderate pain[1]. It is official in USP [2] and BP [3] pharmacopeia. The literature survey reveals several analytical methods for the determination of ANT either single or co-formulated with other drugs including spectrophotometric [4-6], chemometric [7-10], HPLC [11-14], TLC [15-17], GC [18, 19] , capillary zone electrophoresis [20-23] and non aqueous titration [24] methods. While Benzocaine HCl (BEN) is a Benzoic acid, 4-amino, ethyl ester (ethyl 4-aminobenzoate) acid ester in HCl salt used as local anaesthetics for superficial anaesthesia, for the local and temporal relief of pain related, among other disorders, to buccal affections[1]. Besides being official in USP [2] and BP [3] pharmacopeia, BEN was determined by spectrophotometric [25-28], and HPLC methods [14, 29-31]. Also BEN has been analyzed in presence of its degradation product by HPLC [32]. Both aforementioned drugs; Antipyrine and benzocaine, are coformulated as ear drops for the treatment of ear pain caused by otitis media where antipyrine acts as a pain reliever and benzocaine is a numbing agent to treat ear pain [1]. The combination of two or more drugs having complementary action has the advantage of simplicity, convenience and cost-effectiveness. However, as for all chemical mixtures, more sophisticated analytical methods are required for determination and quality control procedures [33]. In the literature only one HPLC method was elaborated for the determination of benzocaine and antipyrine in their binary mixture [14]. Application of this method in our laboratory gave unsatisfactory results, no peak was found for ANT which means that it was completely retained on the column and bad separation of BEN and PABA was obtained as the peak corresponding to PABA was forked and the peak corresponding to BEN was broad. Taking into account these results, the development of validated chromatographic methods for the simultaneous determination of the two drugs in the presence of PABA seemed of primary importance.

2. Experimental

2.1. Instruments

- A liquid chromatography consisted of an "Agilent" HPLC instrument, isocratic pump (ModelG1310Apump, Agilent 1100 series), connected with an UV detector (Model G1314 A, Agilent 1100 series). The injector was a manual Rheodyne injector (Model 7725I, Rohnert Park, CA, USA) equipped with a 20µL injector loop, Agilent (USA). The

instrument was connected to an IBM compatible PC and an HP diskjet 5652 printer. The chromatographic conditions were: Stationary phase: a 5µm, 4.6 x 150 mm I.D. C_8 ZORBAX, 5µm analytical column. Mobile phase: acetonitrile : phosphate buffer solution (Dissolve 1.74 g of dipotassium hydrogen phosphate anhydrous and dilute to a volume of 900 mL with deionised water and adjust to a pH of 5.5 with 85% phosphoric acid) 25:75 (v/v). The mobile phase was filtered through 0.2µm Millipore membrane filter and was degassed for 30 min in an ultrasonic bath prior to use. UV detection was adjusted at 270 nm. The system was operated at ambient temperature. The flow rate was isocratic from 0-4 min (1mLmin^{-1}) then from 4-12 minutes (2 mLmin^{-1}). The samples were filtered also through a 0.2µm membrane filter, and were injected by the aid of a 25µL Hamilton® analytical syringe.

- Camag Linomat 5 autosampler (Switzerland). Camag microsyringe (100µL).
- Camag TLC scanner 3 S/N 130319 with win CATS software, The following requirements are taken into consideration:
  - ❖ Slit dimensions: 5mm×0.2mm.
  - ❖ Scanning speed: 20 mm s^{-1}.
  - ❖ Spraying rate: 10 µLs^{-1}.
  - ❖ Data resolution: 100µm/step.
- Precoated TLC-plates, silica gel 60 F_{254} (20cm × 20 cm, 0.25 mm), E. Merck (Darmstadt-Germany).
- pH-meter, Digital pH/MV/TEMP/ATC meter, Jenco Model-5005(USA).

2.2. Materials and reagents

2.2.1. Pure standard

Benzocaine HCl was kindly supplied by Alexandria Co. for pharmaceuticals, Alexandria, Egypt. Its purity was found to be 100.53±1.442 according to the reported method [32].

Antipyrine (phenazone) was kindly supplied by Amriya for pharmaceutical industries, Alexandria, Egypt. Its purity was found to be 100.07±0.698 according to the reported method [13].

p-aminobenzoic acid (PABA 99.8%) was purchased from Sigma-AldrichChemie GmbH, Taufkirchen, Germany.

2.2.2. Pharmaceutical formulation

Otosept® ear drops are labeled to contain 300 mg of antipyrine and 100 mg of benzocaine HCl in 10 mL of glycerine, It is manufactured by Amriya for pharmaceutical industries, (Alexandria, Egypt). Batch No. 457108[2] (valid dosage form), Batch No. 557105[2] (expired date 9/2013) and Batch No. 457105[2] (expired date 10/2012) were purchased from the Egyptian markets.

2.2.3. Chemicals and reagents

All chemicals used were of analytical grade. De-ionized water: Bidistilled from "Aquatron" Automatic Water Still A4000, Bibby Sterillin Ltd. (Staffordshire, UK). Acetonitrile HPLC grade was obtained from E. Merck, (Darmstadt, Germany) . Hydrochloric acid, dipotassium hydrogen phosphate, phosphoric acid, toluene, acetone, ammonium hydroxide and methanol were obtained from El-Nasr Pharmaceutical Chemicals Co., (Abu-Zabaal, Cairo, Egypt). p-aminobenzoic acid (98%) was purchased from Sigma-Aldrish (chemie GmbH, Germany).

2.3. Standard solution

2.3.1. Stock solution: ANT, BEN (1 $mgmL^{-1}$) and PABA stock solutions (0.5 $mgmL^{-1}$) in mobile phase and methanol for HPLC and TLC-densitometric methods, respectively.

2.3.2. Working solution: ANT, BEN and (PABA) working solutions (0.2 mg mL^{-1}) in mobile phase for HPLC method.

3. Procedures

3.1. Degradation of Benzocaine HCl

Accelerated alkaline and acidic degradation was performed where 0.1 g of pure BEN was accurately weighed and dissolved in 100 mL of 2 mol L^{-1} NaOH and 2 mol L^{-1} HCl. The solution was refluxed at 100°C. Complete hydrolysis was obtained after 30 minutes as investigated by thin layer chromatography (TLC) using mobile phase toluene: acetone: methanol: ammonia (8:3:3:0.1, by volume). The solution was neutralized, evaporated to dryness on boiling water bath and then the residue was dissolved in 50 mL methanol. The obtained solution was filtered on a Whatman filter paper into 100-mL volumetric flask and the volume was completed with methanol to have a concentration of degradation product derived from 1 mg mL^{-1} BEN. The

degradation product was elucidated by its comparison with the purchased PABA where both show similar R_f, t_R, UV and IR spectra.

3.2. HPLC method

3.2.1. Chromatographic conditions

HPLC was carried out at ambient temperature on C_8 column (5µm, 4.6 x 150 mm I.D.). The mobile phase consisted of phosphate buffer pH 5.5: acetonitrile (75:25 v/v) and was filtered using a 0.2µm membrane filter (Billerica, MA) and degassed for 15 min. The mobile phase was delivered at rate of 1mL/min from 0-4 minutes and 2mL/min from 4-12 minutes. The injection volume was 20 µL and the effluent was detected at 270 nm.

3.2.2. Linearity and construction of calibration curves

Accurate aliquots of ANT, BEN and PABA were separately transferred from their respective working standard solutions (0.2 $mgmL^{-1}$) into three separate sets of 10-mL calibrated volumetric flasks to prepare solutions equivalent to 10–100, 5–100 and 5–50 μgmL^{-1} of ANT, BEN and PABA, respectively. 20 µL for each concentration was injected in triplicate. The peak area was recorded then the relative peak area of each concentration was calculated relative to the external standard (10 μgmL^{-1}) ANT, BEN or PABA for ANT, BEN and PABA respectively. The calibration curves were constructed relating relative peak areas to the corresponding concentrations for each drug and the regression equations were computed.

3.3. TLC-Densitometric method

Different aliquots of ANT, BEN and PABA were transferred separately from their standard solutions into three sets of 10-mL volumetric flasks, then the volume was completed with methanol. 10 µL of each solution was spotted as bands of 3 mm width on TLC plates (20x10 cm with 0.25 mm thickness) using a Camag Linomat IV applicator. The bands were applied at 5 mm interval and 10 mm from the bottom and sides. A linear ascending chromatogram was developed to a distance of 8 cm in a chromatographic tank previously saturated for 1 hour with the developing mobile phase consisted of toluene: acetone: methanol: ammonia (8:3:3:0.1 by volume) at room temperature. The peak areas were scanned at 275 nm and the calibration curves were constructed by plotting the integrated

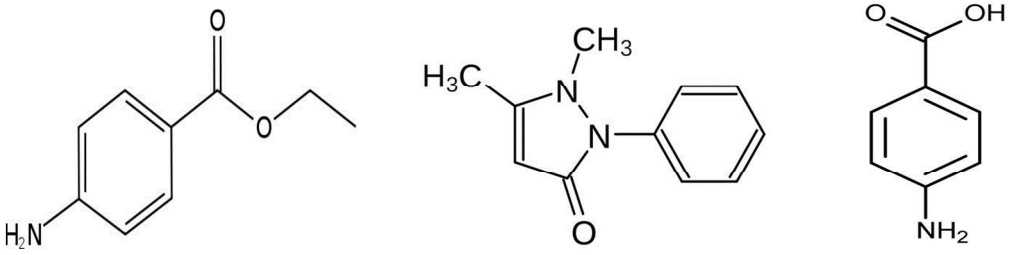
peak areas versus the corresponding concentrations of each compound and the regression equations were computed.

3.4. Application to pharmaceutical formulations

The procedure under linearity and construction of calibration curves was followed using otosept® working solution (0.2 $mgmL^{-1}$ of each compound for HPLC method) and stock solution (1 $mgmL^{-1}$ for TLC method). Concentrations of BEN, ANT and PABA were then calculated from the corresponding regression equations previously computed from which the percentage recoveries were calculated.

4. Results and Discussion

The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drugs substances and products" requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [34]. Moreover; modern quality control of pharmaceuticals concerns with the determination of the main active component and its purity and stability or quantification of impurities. The presence of impurities, even in small amounts, may influence the efficacy and safety of pharmaceutical products and, therefore control of pharmaceutical impurities has become a critical issue to the pharmaceutical industry. BEZ is an ester type drug susceptible to hydrolysis (see Figure 1 for its structure), therefore, it is necessary to study the stability of this drug towards acidic and alkaline hydrolysis process.



Benzocaine
$C_9H_{11}NO_2$,
M.Wt 165.19

Antipyrine
$C_{11}H_{11}N_2O$,
M.Wt 188.23

P-aminobenzoic acid
$C_7H_7NO_2$,
M.Wt 137.14

Figure 1: The structure of benzocaine , antipyrine and p-amionbenzoic acid

On the contrary, ANT resists hydrolysis; so as a result of bad storage, only BEN was expected to be degraded by hydrolysis due to the presence of pharmaceutical excipients [32]. This was confirmed by analyzing expired ear drops containing ANT and BEN by TLC plates where degradation product of BEN (PABA) was detected. To the best of our knowledge, only one analytical method has been reported for the simultaneous determination of ANT and BEN including preliminary stability data of BEN by HPLC [14] method. However, upon analyzing this mixture using the reported mobile phase and C_{18} ODS (30cm x4.6µm, 5 µm) column, bad separation was obtained as shown in, Figure 2. Moreover no validation data were reported for this method, therefore, development of a validated HPLC method that can be used for simultaneous determination of ANT and BEZ in the presence of PABA seemed necessary.

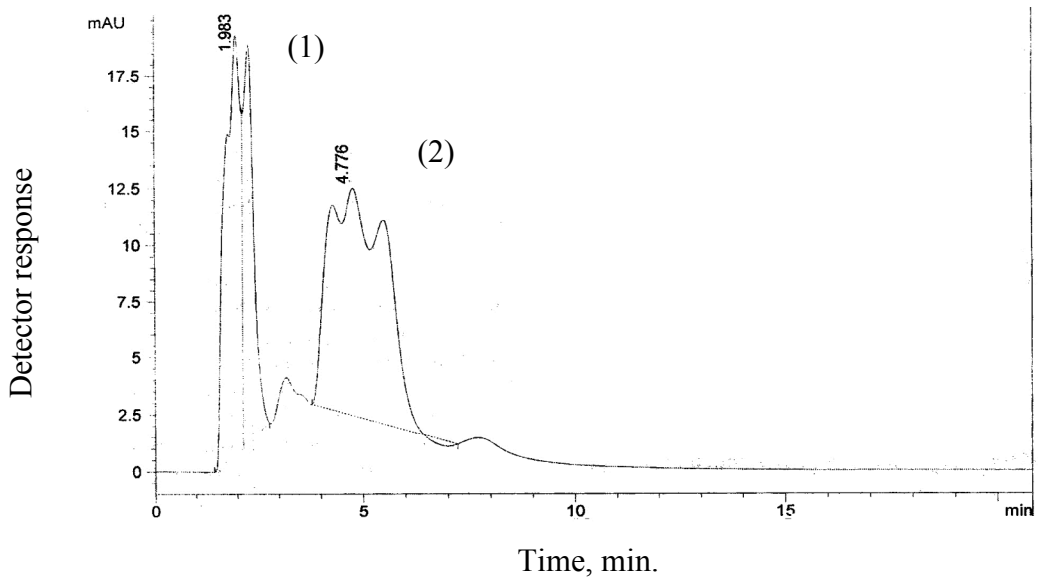


Figure 2: HPLC chromatogram showing mixture of benzocaine HCl (1) with antipyrine (retained on column) and PABA (2) using the reported method [14].

HPLC method

The present work has two main objectives. The first is to report new reliable and validated chromatographic methods as a stability indicating method of BEN where the second is simultaneous determination of ANT and BEN in ear drops. Thus a simple, accurate and

selective HPLC method has been developed and validated for determination of ANT and BEN in the presence of PABA. The procedure was optimized with a view to develop a quantitative and stability indicating method in a convenient time with high resolution of the three components.

Different parameters that affect the efficiency of the chromatographic separation have been tested and optimized in a trial to obtain maximum separation of the cited components. The first parameter that was investigated is the influence of the amount of organic modifier on the peak shape and retention time of the cited components. The use of either acetonitrile: methanol: H_2O or acetonitrile: phosphate buffer in different ratios resulted in bad separation between ANT and PABA. However, a single sharp peak was obtained when the acetonitrile: phosphate buffer pH5.5 ratio adjusted to (25:75). This ratio has been selected as the mobile phase composition for this method. Increasing acetonitrile concentration led to inadequate separation of ANT and PABA. At lower acetonitrile concentration (<25%), separation occurred but with excessive tailing and longer retention time for BEN .

Variation in the pH of the mobile phase has a great effect on the separation of the cited compounds. At pH lower than 5; PABA eluted at a retention time of 0.7 minutes, while at pH above 7, ANT and PABA appeared as a single broad peak. Moreover the peak corresponding to BEN eluted closer to that of ANT. Improvement in separation was observed at pH 4.5–6.5 where optimum resolution with reasonable retention time was achieved at pH 5.5.

Several wavelengths were tested (254, 270 and 288 nm), the most suitable wavelength was 270 nm at which high sensitivity of both ANT and BEN was obtained. The mobile phase was delivered at different rates (1, 1.5 and 2 $mLmin^{-1}$), the optimum was 1 $mLmin^{-1}$ for 4 min then changed to 2 $mLmin^{-1}$ which provides maximum separation with minimum run time because if apply 1 $mLmin^{-1}$ for all experiment BEN will appear at 24 min.

HPLC chromatogram is illustrated in Figure 3 where complete separation of the three compounds was noticed. The retention time for PABA, ANT and BEN were found to be 1.91±0.05, 3.78±0.06 and 10.05±0.08 min, respectively. Table 1 shows parameters of system suitability of the proposed HPLC method.

The calibration curves were constructed by plotting the relative peak areas versus the concentrations in the range of 10–100 µg mL^{-1} for ANT, 5–100 µg mL^{-1} for BEN and 5-50 µg mL^{-1} for PABA. ANT, BEN and PABA concentrations were calculated from the following

regression equations:

For ANT, $Y_1 = 0.1002\ C1 - 0.0113$ $r_1 = 0.9998$

For BEN, $Y_2 = 0.0812\ C2 - 0.0900$ $r_2 = 0.9999$

For PABA, $Y_3 = 0.0101\ C_3 - 0.0173$ r3 = 0.9995

Where Y_1, Y_2 and Y_3 are the relative peak areas, C_1 , C_2 and C_3 are concentrations in µg mL^{-1}, and r_1, r_2 and r_3 are the correlation coefficients of ANT, BEN and PABA, respectively.

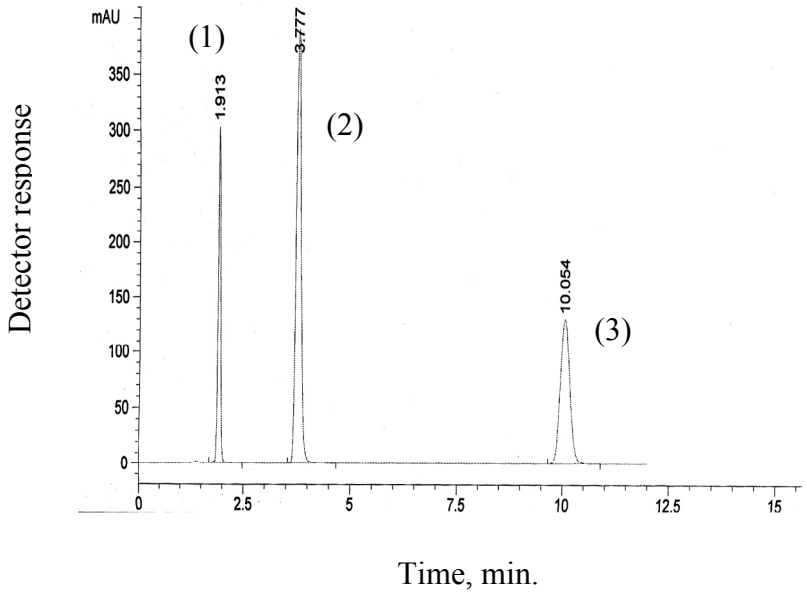


Figure 3: HPLC chromatogram of 10 µgmL^{-1} of BEN degradation product (PABA) (1), 60 µgmL^{-1} of antipyrine (2) and 20 µgmL^{-1} benzocaine HCl (3) at 270 nm.

TLC-Densitometric method

TLC is a well recognized routine analytical technique that offers several advantages such as disposable stationary phase, static detection free of time constraints, storage of chromatographic information, wider range of detection possibilities, the use of small volumes of solvents, minimum sample cleanup, and simultaneous analysis of several components in a short time, which in turn reduce analytical run times [35]. This technique offers a simple way to quantify the separated bands of different compounds by measuring their respective optical density. The amounts of compounds are determined by comparing to a standard reference materials chromatographed simultaneously under the same condition [35]. Samples are applied on TLC

plate as bands using Linomat IV sample applicator which is more advantageous than spotting as it provides better separation, minimizes zones tailing and increases linearity range. Moreover, application of the different volumes from one solution as bands gives the same concentration-response curve as obtained by application of equal volumes of solutions of different concentrations. This correlation is absent when sample solution is applied as spot. [37]. The only disadvantage of band application is that fewer samples can be accommodated on a given plate. To improve separation of bands, it was necessary to investigate the effect of different variables including mobile phase, band dimensions, scanning wavelength and slid dimensions of scanning light beam.Different developing systems of different composition and ratios were tried for separation, e.g. ethylacetate: chloroform: ammonium hydroxide (8: 2: 0.4, by volume), ethylacetate: chloroform: glacial acetic acid (8:2:0.2, by volume), ethyacetate: methanol: ammonium hydroxide (8:1:1, by volume) and chloroform: acetone: methanol: ammonium hydroxide (5:5: 2: 0.2, by volume). The best mobile phase was Toluene–acetone– methanol: ammonium hydroxide (8:3:3:0.1, by volume). The selected mobile phase allows good separation between ANT, BEN and PABA with good R_f values without tailing of the separated bands, Figure 4.

Different band dimensions were tested in order to obtain sharp and symmetrical separated peaks. The optimum band width chosen was 3 mm and the inter-space between bands was 5 mm. Different scanning wavelengths were tried, where 275 nm was the best wavelength for both drugs at which peaks were sharper and symmetrical and minimum noise was obtained, at this wavelength maximum sensitivity for both drugs was obtained. The slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried, where 5 mm x 0.2 mm proved to be the slit dimension of choice which provides highest sensitivity. Following USP [2] system suitability tests, parameters were calculated to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis. Parameters including resolution (R_s), peak symmetry, capacity factor (K') and selectivity factor (α) were calculated using 5 µg/band of both drugs. The resolution is always above two, the selectivity more than one and an accepted value for symmetry factor was obtained for each component, as shown in Table 2.

This method is based on the difference in the R_f values of ANT (R_f = 0.48), BEN (R_f = 0.64), and

PABA (R_f = 0.21), as shown in Figure 4.

The calibration curves were constructed by plotting the integrated peak areas versus the corresponding concentrations in the range of 1.25–8.75, 2.5-8.75 and 1-3 µg/band for ANT, BEN and PABA, respectively. The concentrations of ANT, BEN and PABA were calculated from the following regression equations:

For ANT, $A_1 = 0.1316 \times 10^4 C_1 + 0.6085 \times 10^4$ $r_1 = 0.9995$

For BEN, $A_2 = 0.2159 \times 10^4 C_2 + 1.7270 \times 10^4$ $r_2 = 0.9998$

For PABA $A_3 = 6.0253 \times 10^4 C_3 + 11.0690 \times 10^4$ $r_3 = 0.9996$

Where A_1, A_2 and A_3 are the integrated peak area, C_1, C_2 and C_3 are the concentration in µg/ band, and r_1, r_2 and r_3 are the correlation coefficients of ANT, BEN and PABA, respectively.

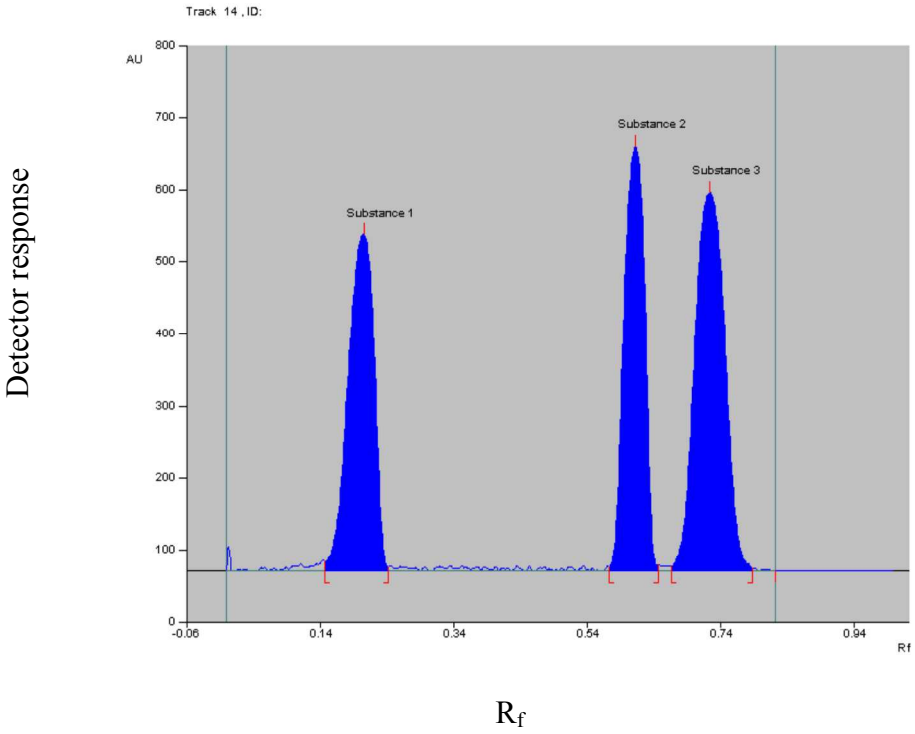


Figure 4: Thin layer chromatogram of (1) P-aminobenzoic acid at R_f = 0.21 ± 0.05, (2) antipyrine at R_f = 0.48± 0.03 and benzocaine HCl at R_f= 0.64 ± 0.02 using a mobile phase toluene: acetone: methanol: ammonium hydroxide (8: 3:3: 0.1, by volume).

4.4. Application of the proposed methods to the pharmaceutical formulations

The suggested methods were applied for the determination of ANT, BEN and PABA(if any) in their valid and expired pharmaceutical formulations. In valid otosept® ear drop; only ANT

and BEZ were detected, while in expired one there was a remarkable decrease in the peak corresponding to BEZ with the appearance on a new one corresponding to PABA. Results are summarized in Table 3. The validity of the suggested methods was further assessed by applying the standard addition technique as shown in Table 3. Method validation was performed according to USP guidelines [2] for the proposed methods and Table 4 shows results of accuracy, repeatability and intermediate precision of the methods.

4.5. Specificity and selectivity

The selectivity of the method was demonstrated by good separation of the studied components, Figures 3&4. Also, the absence of any peaks at the retention times of the studied drugs and the good results obtained on applying the method to otosept® ear drops, the results presented in Table 3 prove that there was no interference from excepients.

4.6. Statistical analysis

Results of the suggested methods for determination of ATN and BEN were statistically compared with those obtained by applying the reported methods [13, 32] for ANT and BEN, respectively. The calculated *t*- and *F*-values [38] were found to be less than the corresponding theoretical ones, confirming good accuracy and excellent precision as given in Table 5.

Conclusion

In this work two stability indicating methods were developed for simultaneous determination of antipyrine and benzocaine HCl in the presence of reported BEN degradation product (PABA).

The advantage of TLC-densitometric method is that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering analysis time and cost per analysis and provides high sensitivity and selectivity. While HPLC has the advantage of being highly selective compared with other published HPLC methods which cannot separate ANT and BEN in the presence of PABA.

The published stability indicating method for determination BEN [32] not suitable for determination of ANT. The suggested methods are found to be simple, accurate, selective and equally sensitive with no significant difference of the precision compared with the

reported methods. They could be applied for routine analysis and quality control of pure drugs or pharmaceutical formulations.

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Table 1: Parameters required for the system suitability test of HPLC method.

Parameter	p-aminobenzoic acid	Antipyrine	Benzocaine HCl	Reference values[39]
Resolution	6.7	10.5		R > 1.5
T (tailing factor)	≈1	≈1	1.15	T = 1 for a typical symmetric peak
α (relative retention time)	1.97	2.66		> 1
K (column capacity)	0.91	2.78	9.05	1-10 acceptable
N (column efficiency)	1243	2147	2146	Increases with efficiency of the separation
HETP = L (length of column in cm)/N	1.2×10^{-2}	6.99×10^{-3}	6.99×10^{-3}	The smaller the value, the higher the column efficiency

Where 1, 2 and 3 are antipyrine, benzocaine HCl and p-aminobenzoic acid, respectively.

Table 2: Parameters of system suitability of the developed TLC-densitometric method for Antypyrine, benzocaine HCl and BEN degradation product (PABA).

parameter	p-aminobenzoic acid	Antipyrine	Benzocaine HCl
Symmetry factor	1.07	1.10	1.06
Resolution (R_s)	5.06		1.17
Capacity factor (K')	3.76	1.08	0.56
Selectivity (α)	3.48		1.92

Table 3: Analysis of Otosept®* ear drops and application of the standard addition technique to the determination of benzocaine HCl and antipyrine in Otosept®* ear drops by the proposed HPLC and TLC-densitometric methods.

The proposed method	**Otosept® ear drops**		**Standard addition technique**		
	Recovery ± SD		**Pure added (µgmL^{-1})**	**Pure found (µgmL^{-1})**	**Recovery %**
High performance liquid chromatography	Antipyrine	99.42±1.022[a]	10	9.82	98.20
		99.53±1.105[b]	30	30.24	100.80
		100.55±0.975[c]	40	40.23	100.58
	Mean ± SD				99.86±1.442
	Benzocaine	101.62±0.687[a]	10	10.01	100.10
		86.31±0.327[b]	20	20.38	101.94
		78.16±1.309[c]	40	40.12	100.30
	Mean ±SD				100.78±1.009
	(PABA)	13.37±0.353[b]			
		22.67±1.176[c]			
The proposed method	**Otosept® ear drops**		Standard addition technique		
	Recovery ± SD		Pure added (µg/band)	Pure found (µg/band)	Recovery %
TLC-densitometric method	Antipyrine	100.55±1.946[a]	2.0	2.01	100.50
		99.75±1.656[b]	3.0	3.03	101.00
		100.35±0.974[c]	4.0	3.99	99.50
	Mean ± SD				100.33±0.763
	Benzocaine	99.92±1.874[a]	2.0	1.98	99.00
		87.05±0.943[b]	3.0	2.95	98.33
		76.77±0.633[c]	4.0	4.02	100.50
	Mean ±SD				99.28±1.109
	(PABA)	12.97±0.663[b]			
		23.09±0.995[c]			

[a]Valid dosage form Batch No. 457108 [2]

[b]expired dosage form Batch No. 557105 [2], the expired date has been passed by 6 months

[c]expired dosage form Batch No. 457105 [2], the expired date has been passed by 17 months*Average of three determination

Table (4): Results of assay validation parameters of the proposed methods for determination of Antiyprine and Benzocaine HCl in pure form.

Parameters	HPLC			Densitometric method		
	Antipyrine	Benzocaine HCl	PABA	Antipyrine	Benzocaine HCl	PABA
Range	10-100 µg mL^{-1}	5-100 µg mL^{-1}	5-50 µg mL^{-1}	1.25-8.75 µg/band	2.5-8.75 µg/band	1-3 µg/band
Linearity						
Slope	0.1002	0.0812	0.1001	0.1316 $x10^4$	0.2159$x10^4$	6.0253 $x10^4$
Intercept	-0.0113	0.0900	0.0173	0.6085 $x10^4$	1.7270$x10^4$	11.0690$x10^4$
(r)	0.9998	0.9999	0.9997	0.9995	0.9998	0.9996
Accuracy (mean ±SD)	100.22 ±1.375	99.77 ±1.089	100.42 ±1.605	99.93 ±0.985	100.08 ±1.041	99.90 ±1.590
LOD	5.0 µgmL-1	2.50 µgmL-1	2.00 µgmL-1	1.00 µg/band	1.25 µg/band	0.5 µg/band
LOQ	10.0 µgmL-1	5.0 µgmL-1	5.0 µgmL-1	1.25 µg/band	2.50 µg/band	1.0 µg/band
Specificity and Selectivity		The method showed good separation of the two drugs and the degradation product				
Precision (RSD%)						
Repeatability**	0.330-0.369-0.505	1.046–0.244–0.267		0.219-0.121-0.392	0.550-0.255-0.910	
Intermediate precision**	1.830-1.141-1.317	1.567-0.499–0.665		1.084-1.089-1.104	0.892-1.164-1.368	

* **Correlation coefficient**

**The intraday precision (n=9), average of three different concentrations repeated three times within day. The interday precision (n=9), average of three different concentrations repeated three times in three successive days.

Table (5): Statistical comparison of the results obtained by the proposed methods and the Official method for the determination of pure antipyrine and benzocaine HCl in pure powder form.

Items	Antipyrine			Benzocaine HCl		
	HPLC method	TLC densitometric	Reported method [13]	HPLC method	TLC densitometric	Reported Method[32]
Mean	100.22	99.93	100.07	99.77	100.08	100.53
S. D.	1.375	0.985	0.698	1.089	1.041	1.442
n	6	7	5	7	6	5
Variance	1.89	0.985	0.487	1.186	1.089	2.079
Student's t test	0.222 (2.262)	0.271 (2.228)		1.044 (2.228)	0.601 (2.262)	
F test	3.88 (6.26)	1.99 (6.16)		1.75 (4.53)	1.92 (5.19)	

Figure between parenthesis are the corresponding tabulated values (p=0.05)