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# Determination of naphazoline HCl, pheniramine maleate and their official impurities in eye drops and biological fluid rabbit aqueous humor by a validated LC-DAD method†

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A simple RP-HPLC-DAD method was developed and validated, as per the ICH guidelines, for simultaneous determination of naphazoline HCl (NPZ) & pheniramine maleate (PHN) along with three of their official impurities. Chromatographic separation was performed on a hypersil ODS column (5 mm, 250–4.6 mm i.d.) with isocratic elution using phosphate buffer pH 6.0: acetonitrile (70 : 30, v/v) as mobile phase, at a flow rate of 1.0 mL min<sup>-1</sup> and UV detection at 260.0 nm. The developed method was found to be linear over the concentration ranges of 5.00–45.00 µg mL<sup>-1</sup> for NPZ and NPZ impurity B and 10.00–110.00 µg mL<sup>-1</sup>, 10–70 µg mL<sup>-1</sup> and 10–120 µg mL<sup>-1</sup> for PHN, and PHN impurity A and B, respectively, with correlation coefficient values <0.999 for the five cited compounds. The method was confirmed to be accurate, robust and precise with RSD >2.0%. LOD and LOQ values for the five cited compounds were calculated. Moreover, the method was also validated in rabbit aqueous humor as per the US food and drug administration (FDA) bioanalytical validation guidelines. Finally, the proposed method was applied for the analysis of the two drugs along with their impurities in dosage form and spiked aqueous humor samples.

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## 1 Introduction

With growing pharmaceutical industries, and development of modern analytical techniques, more concern is given by the pharmacopoeias and regulatory authorities to drug purity and impurity detection. Drug purity is an essential factor for ensuring drug safety and quality.<sup>1</sup> Moreover, some of the impurities may contribute to the drug side effects, and thus development of an analytical method for their determination is a must.<sup>2</sup>

According to the world allergy organization, allergic conjunctivitis is considered as a broad group of allergic conditions including; inflammation of conjunctiva, itching, edema and increased lacrimation.<sup>3</sup> In high frequency, allergic rhinitis and asthma may also occur.<sup>4</sup> Generally, ocular allergies affect about 5–22% of the population.<sup>5</sup> There are different types of mast cell stabilizer and antihistaminic drugs used to control

those allergies.<sup>6</sup> In addition, decongestant drugs play an important role through reducing swelling and edema of conjunctiva.<sup>7</sup>

Naphazoline HCl (NPZ), used as a decongestant drug, has a sympathomimetic effect on  $\alpha$  adrenergic receptors. It acts on those receptors in the arterioles of the conjunctiva, resulting in reducing swelling and edema.<sup>8</sup> It is chemically designated as [2-(naphthalen-1-ylmethyl)-4, 5-dihydro-1H-imidazole; HCl]. It is an official drug in the US (USP)<sup>9</sup> and British (BP)<sup>10</sup> pharmacopoeias where its assay was performed by HPLC methods. Moreover, BP reports four official impurities; A, B, C and D. Literature review revealed that NPZ has been determined either in bulk powder or in presence with other drugs using several techniques including; spectrophotometry,<sup>11–20</sup> HPLC,<sup>21–32</sup> TLC<sup>33</sup> and capillary electrophoresis.<sup>34–40</sup>

Pheniramine maleate (PHN) is an antihistaminic drug with anticholinergic properties. It binds to histamine H1 receptors, leading to inhibiting phospholipase A2, reducing cyclic GMP levels, and decreasing histamine. PHN is commonly found in eye drops, which are used for treatment of allergic conjunctivitis.<sup>41</sup> Chemically, it is [(Z)-but-2-enedioic acid; N,N-dimethyl-3-phenyl-3-pyridin-2-ylpropan-1-amine]. HPLC methods are used for its assay in USP and BP. Two impurities (A and B) were specified in BP for PHN.<sup>9,10</sup> On the other hand, it has been determined in its bulk powder or in the presence of other drugs

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using several methods, such as spectrophotometric,<sup>42,43</sup> HPLC,<sup>30–32,44–51</sup> capillary electrophoretic<sup>40,52</sup> and titrimetric.<sup>53</sup>

NPZ and PHN have been co-formulated in eye drops for treatment of eye inflammation and allergic conjunctivitis. This combination has found to be more effective than using each drug separately.<sup>54</sup> Reviewing the previous literature reveals that NPZ and PHN have been simultaneously determined by HPLC<sup>30–32</sup> and capillary electrophoresis.<sup>40</sup> However, there is no reported analytical method dealing with their determination along with their official impurities. Therefore, the aim of this work was to analyze NPZ and PHN along with three selected impurities, namely; NPZ impurity B, PHN impurity A and B, using a validated RP-HPLC-DAD method. The method was then applied for the determination of the five compounds (Fig. 1) in their quinary mixture, eye drops and spiked rabbit aqueous humor (for the purpose of determining their extraction efficiency). In this study, animal models were preferable, as they offer more flexibility in obtaining sufficient amounts of ocular fluid without causing a tissue damage. Rabbits were the animals of choice since they are small, easy to handle, low cost, and have a similar eye size to humans.<sup>55</sup>

## 2 Experimental

### 2.1. Instruments

The HPLC-DAD system consisted of pump with different flow rates (model Waters Alliance 2695) equipped with a photodiode array detector (DAD) and a 100 mL injection loop. A hypersil ODS column (5 mm, 250–4.6 mm i.d.) was used as stationary phase. The samples were injected by auto sampler system. pH of the solutions was adjusted by a pH meter (Mettler Toledo MA 235).

### 2.2. Materials and chemicals

**2.2.1. Pure standard.** The two drugs; NPZ, PHN and dexamethasone internal standard (IS) were kindly provided by Eva pharma pharmaceutical company, Cairo, Egypt. Purities of NPZ and PHN were checked and found to be  $100.12 \pm 0.10\%$  and  $99.58 \pm 0.12\%$ , respectively, according to the BP methods.<sup>10</sup> NPZ impurity B, PHN impurity A and B were purchased from Alfa Aesar Company (Germany). Their respective certified potency values were 99.00%, 100.30%, and 99.70%.

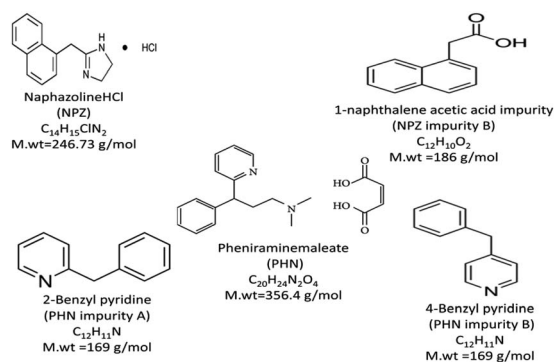


Fig. 1 Chemical structures of the five studied compounds.

**2.2.2. Pharmaceutical dosage form.** Naphcon-A® eye drop is the product of Alcon laboratories INC, Novartis Company. It is labelled to contain 0.25 mg NPZ and 3.0 mg PHN per one mL. Batch no. H13949-0615 was obtained from local drug pharmacy.

**2.2.3. Chemicals and reagents.** All chemicals used were of analytical reagent grade and solvents were of HPLC grade; acetonitrile (Merck, Germany), double distilled deionized water (Otsuka, Cairo, Egypt), triethylamine (TEA) (Sigma-Aldrich, Belgium), phosphate buffer solution pH 6.0 (0.8 mL (0.16%) orthophosphoric acid and 0.4 mL (0.08%) TEA to 500 mL double-distilled deionized water, then pH was adjusted to 6.0 using 10% KOH.<sup>56</sup> A mobile phase of phosphate buffer pH 6.0: acetonitrile, 70.0 : 30.0 v/v, was prepared.

**2.2.4. Rabbit aqueous humor.** Ten animals of New Zealand male rabbits (2.5–3.0 kg), purchased from the National Research Center (Giza, Egypt), were used in this study. Prior to extraction of aqueous humor, two drops of oxybuprocaine HCl local anesthetic were applied to rabbits' eyes. After that, the aqueous humor immediately removed from the anterior chamber of each eye using a 26-gauge needle attached to tuberculin syringe. The procedure was repeated to collect sufficient volume for the required analyses. The obtained samples were then stored at  $-20\text{ }^{\circ}\text{C}$  until carrying out the experiments.<sup>57</sup> All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals protocol, and approved by Research Ethics Committee-Faculty of Pharmacy, Cairo University.

### 2.3. Solutions

Stock standard solutions of  $1.0\text{ mg mL}^{-1}$  of the five studied compounds were separately prepared by transferring 10.00 mg of each substance into 10 mL volumetric flasks. Dissolved and completed to the mark using mobile phase.

### 2.4. Procedures

**2.4.1. Chromatographic conditions.** Isocratic elution was applied using mobile phase consisting of phosphate buffer pH 6.0 and acetonitrile (70.0 : 30.0 v/v). The drugs were separated using hypersil ODS column (5 mm, 250–4.6 mm i.d.) with a flow rate  $1.0\text{ mL min}^{-1}$ . The prepared buffer was filtered through  $0.45\text{ }\mu\text{m}$  membrane filter. The injection volume was  $10.0\text{ }\mu\text{L}$  and the chromatograms were scanned from 200.0–400.0 nm by the aid of DAD and it was found that at 260.0 nm optimum resolution was achieved.<sup>58</sup>

#### 2.4.2. Analysis of eye drops

**2.4.2.1. Calibration curves.** Calibration curves of the two drugs and their impurities were constructed by transferring different aliquots from their stock standard solutions equivalent to 50.0–450.0  $\mu\text{g}$  for NPZ and NPZ impurity B, 100.0–1100.0  $\mu\text{g}$  for PHN, 100.0–700.0  $\mu\text{g}$  for PHN impurity A, and 100.0–1200.0  $\mu\text{g}$  for PHN impurity B into five sets of 10 mL volumetric flasks. The volumes were then completed to the mark using mobile phase. The detailed conditions previously described were applied. Linearity of the studied drugs was tested by plotting the integrated peak areas obtained against the used concentrations, then regression equations were computed.



**2.4.2.2. Assay of laboratory prepared mixtures.** Solutions containing different volumes of the two drugs along with their three impurities were prepared by serial dilutions from their corresponding stock standard ones into 10 mL volumetric flasks. The volume of each flask was then completed to the mark with mobile phase. They were analyzed using the previous chromatographic conditions.

**2.4.2.3. Application to pharmaceutical dosage form.** From Naphcon-A® eye drop, 1.0 mL aliquot was accurately transferred into a 25 mL volumetric flask. 15.0 mL of mobile phase was added and the flask was sonicated for 15 min. The volume was then completed to mark using the same solvent. The final concentration obtained was  $10 \mu\text{g mL}^{-1}$  for NPZ and  $120 \mu\text{g mL}^{-1}$  for PHN. This procedure was repeated four times. The previously described chromatographic conditions were followed for NPZ and PHN determination.

### 2.4.3. Analysis in rabbit aqueous humor

**2.4.3.1. Calibration curves.** Calibration curves were constructed in the range of  $5.0\text{--}45.0 \mu\text{g mL}^{-1}$  for NPZ and NPZ impurity B and  $10.0\text{--}110.0$ ,  $10.0\text{--}70.0$  and  $10.0\text{--}120.0 \mu\text{g mL}^{-1}$  for PHN, PHN impurity A and B, respectively, by transferring 1.0 mL aliquots from their standard solutions to separated test tubes, each containing 1.0 mL aqueous humor and  $100.0 \mu\text{g IS}$ . The samples were vortexed for 1 min. 3.0 mL methanol was then added for protein precipitation. The supernatants were finally separated, evaporated to dryness and reconstituted using 1.0 mL aliquot of mobile phase. The detailed conditions previously described were applied. Linearity of the studied drugs was tested by plotting the ratio of the integrated peak area to the peak area of IS obtained against the used concentrations, and then regression equations were computed.

**2.4.3.2. Assay of spiked rabbit aqueous humor samples.** Solutions containing different concentrations the two drugs and their impurities were prepared by transferring accurately different aliquots from their respective stock solutions into a set of 10 mL volumetric flasks. The volumes were completed to the mark using mobile phase. From the previously prepared solutions, aliquots of 1.0 mL were transferred to test tubes separately.  $100.0 \mu\text{g IS}$  and 1.0 mL aqueous humor were then added to each test tube. The samples were vortexed for 1 min and 3.0 mL methanol was then added for protein precipitation. Finally, the obtained solutions were vortexed vigorously for 5 min followed by centrifugation at 4500 rpm for 15 min. The supernatants were separated and evaporated to dryness using nitrogen steam. The residues were reconstituted using 1 mL aliquot of mobile phase and the solutions were passed through  $0.45 \mu\text{m}$  membrane filter.<sup>59</sup> The final concentration range obtained was  $10.00\text{--}45.00$ ,  $20.00\text{--}90.00$ ,  $15.00\text{--}45.00$ ,  $25.00\text{--}70.00$  and  $20.00\text{--}120.00 \mu\text{g mL}^{-1}$  for NPZ, PHN, NPZ impurity B, PHN impurity A and B, respectively. The prepared solutions were analyzed adopting the previously mentioned chromatographic conditions.

## 3 Results and discussion

### 3.1. Method optimization

In order to achieve optimum separation, several trials have been performed, as using phosphate and/or acetate buffers with

different pH values (3.0, 5.0 and 6.0). It was found that mobile phase with isocratic elution containing phosphate buffer pH 6.0 and acetonitrile with ratio (70.0 : 30.0 v/v) gave the best resolution between the analyzed components. It worth noting that pH 6.0 was the one that enhance system suitability parameters regarding number of theoretical plates and tailing factor. TEA modifier was added for improving resolution and enhancing the symmetry of the separated peaks.<sup>60</sup> After testing different types of columns including CN, C8 and C18 ones, the most separation efficiency and the best system suitability parameters were obtained upon using hypersil ODS column (5  $\mu\text{m}$ , 250–4.6 mm i.d.) with run time less than 30 min using  $1.0 \text{ mL min}^{-1}$  flow rate. DAD was adjusted at different wave lengths from 200.0 nm to 400.0 nm and 260.0 nm was chosen as an optimum wavelength for detection of the studied compounds. Chromatograms obtained during method optimization are shown in (Fig. S1–S5, ESI†).<sup>61</sup> The  $t_{\text{R}}$  values were  $3.82 \pm 0.1$ ,  $6.04 \pm 0.1$ ,  $7.87 \pm 0.1$ ,  $21.68 \pm 0.1$  and  $27.28 \pm 0.1$  min for PHN, PHN impurity B, NPZ, PHN impurity A and NPZ impurity B respectively (Fig. 2). To assess the system suitability, various parameters were studied as; resolution, tailing factor, retention time, selectivity, column efficiency and height equivalent to theoretical plate. The obtained results were tabulated in Table 1.

### 3.2. Method validation for dosage form analysis

For analysis of eye drops, validation of the proposed chromatographic method was conducted according to ICH guideline.<sup>62</sup>

**3.2.1. Linearity and range.** NPZ and NPZ impurity B show linear relationship between the peak area and the corresponding concentrations in range of  $5.0\text{--}45.0 \mu\text{g mL}^{-1}$ . PHN, PHN impurity A and B show their linearity at concentrations  $10.0\text{--}110.0$ ,  $10.0\text{--}70.0$  and  $10.0\text{--}120.0 \mu\text{g mL}^{-1}$ , respectively. The analyses were performed as described before under procedure section. The obtained result of regression equations, LOD and LOQ along with their relative standard errors were summarized in Table 2.

**3.2.2. Accuracy.** Accuracy was determined on pure samples by applying the proposed chromatographic method on different

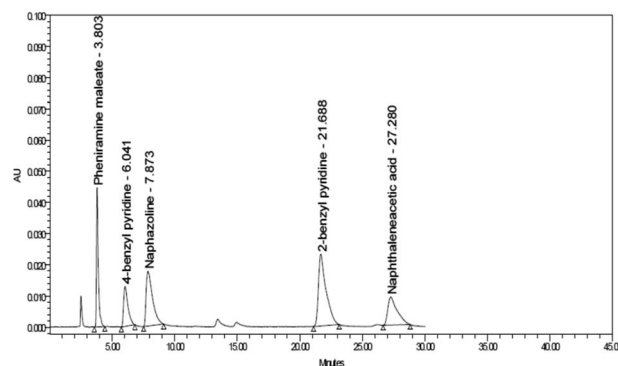


Fig. 2 HPLC chromatograms of NPZ ( $35.00 \mu\text{g mL}^{-1}$ ), PHN ( $90.00 \mu\text{g mL}^{-1}$ ) and three of their selected impurities; NPZ impurity B ( $35.00 \mu\text{g mL}^{-1}$ ), PHN impurity A ( $50.00 \mu\text{g mL}^{-1}$ ) and PHN impurity B ( $90.00 \mu\text{g mL}^{-1}$ ) using a hypersil ODS column (5 mm, 250–4.6 mm i.d.), mobile phase of pH 6.0 phosphate buffer: acetonitrile (70 : 30, by volume), flow rate of  $1.0 \text{ mL min}^{-1}$  and detection at 260 nm.



Table 1 System suitability parameters for the proposed HPLC-DAD method

Parameter	PHN	PHN imp-B	NPZ	PHN imp-A	NPZ imp-B	Reference values
$t_R$ (min)	3.80	6.04	7.87	21.68	27.28	NA
Resolution ( $R_s$ )	NA	4.73	2.29	11.99	4.02	$R_s > 1.5$
Tailing factor ( $T$ )	1.26	1.55	1.68	1.52	1.72	NA
Retention factor ( $k'$ ) <sup>a</sup>	1.11	2.36	3.37	11.04	14.16	$1 < k' < 10$
Selectivity factor ( $\alpha$ ) <sup>b</sup>	NA	2.13	1.43	3.28	1.28	$\alpha > 1$
Column efficiency ( $N$ ) <sup>c</sup>	3192.03	2533.94	2260.58	6867.68	7601.12	NA
Height equivalent to theoretical plate (mm)	0.08	0.10	0.11	0.04	0.03	NA

<sup>a</sup> Retention factor ( $K$ ) =  $(t_R - t_m)/t_m$ . <sup>b</sup> Calculation of  $\alpha = K_2/K_1$ . <sup>c</sup> Column efficiency ( $N$ ) =  $16(t_R/w)^2$ , where  $w$  is the peak width and  $t_R$  is the retention time.

concentrations; the obtained results (recoveries) were tabulated in Table 2.

### 3.2.3. Precision

**3.2.3.1. Repeatability.** From linearity range, three different concentrations were chosen. 10.0, 25.0, 40.0  $\mu\text{g mL}^{-1}$  for NPZ and NPZ impurity B, 20.0, 45.0, 70.0  $\mu\text{g mL}^{-1}$  for PHN, PHN impurity A and B. Those concentrations were analyzed three times intra-daily using the proposed HPLC-DAD method. The result expressed by relative standard deviations (RSD%) in Table 2, showing high repeatability and low deviations.

**3.2.3.2. Intermediate precision.** The three previously chosen concentrations were analyzed three times using the previously mentioned method inter-daily on three different days. Acceptable values of RSD% are shown in Table 2.

**3.2.4. Specificity.** The method was proved to be specific by analyzing different laboratory prepared mixtures with different ratios of the five cited drugs. The recovery percentage and RSD% are shown in Table 2.

**3.2.5. Robustness.** Robustness was assessed by studying the effect of small deliberates occurred in the chromatographic conditions of the HPLC-DAD method on peak area and resolution. The method was found to be robust to small changes in chromatographic conditions as percentage of organic solvent (30.0  $\pm$

1.0%), pH ( $6.0 \pm 0.02$ ) and flow rate ( $1.0 \pm 0.05 \text{ mL min}^{-1}$ ). The relative standard deviations are presented in Table 2, showing low deviations and high repeatability.

### 3.3. Application to pharmaceutical dosage form

NPZ and PHN were analyzed by the proposed HPLC-DAD method without any interference from their eye drops additives. Standard addition technique was applied with appropriate recoveries; the results are shown in Table 3.

### 3.4. Statistical analysis

The results obtained from NPZ and PHN analysis in presence of their official pharmacopeial impurities (NPZ impurity B, PHN impurity A and B) were statistically compared with their respective BP methods of analysis.<sup>10</sup>The calculated  $t$  and  $F$  are shown in Table 4.

### 3.5. Method validation for analysis in rabbit aqueous humor

The validation of the proposed method in rabbit aqueous humor was performed according to U.S. FDA bioanalytical validation guidelines.<sup>63</sup>

**3.5.1. Selectivity.** The method selectivity was proved by comparing the chromatograms of the blank aqueous humor

Table 2 Regression and validation parameters for determination of the studied drugs by the proposed HPLC-DAD method

Parameter	PHN	PHN imp-B	NPZ	PHN imp-A	NPZ imp-B
Range	10–110 $\mu\text{g mL}^{-1}$	10–120 $\mu\text{g mL}^{-1}$	5–45 $\mu\text{g mL}^{-1}$	10–70 $\mu\text{g mL}^{-1}$	5–45 $\mu\text{g mL}^{-1}$
Slope	3564.18	2988.87	16 867.08	14 527.48	9845.23
Intercept	48 167.40	3498.28	–45321.53	–12384.81	–16524.53
SE of the slope	34.86	34.43	181.62	174.23	117.56
SE of the intercept	2341.06	2509.26	5110.20	7627.92	3307.67
Specificity (mean $\pm$ SD) <sup>a</sup>	99.27 $\pm$ 1.52	100.31 $\pm$ 1.50	99.75 $\pm$ 1.23	100.06 $\pm$ 1.41	99.17 $\pm$ 1.74
Accuracy (recovery%)	99.62	99.02	100.35	100.37	99.94
<b>Precision (RSD%)</b>					
Repeatability	0.26	1.31	0.51	0.21	1.55
Intermediate precision	0.80	0.96	0.97	0.74	0.50
Robustness	1.20	1.49	0.98	0.77	1.15
LOD ( $\mu\text{g mL}^{-1}$ )	3.10	3.00	1.29	1.98	1.43
LOQ ( $\mu\text{g mL}^{-1}$ )	9.40	9.11	3.90	5.99	4.33
Correlation coefficient ( $r$ )	0.9992	0.9990	0.9991	0.9990	0.9990

<sup>a</sup> Average of determinations of seven laboratory prepared mixtures.





**Table 3** Determination of NPZ, PHN in their dosage form and application of standard addition technique using the proposed HPLC-DAD method

Naphcon-A® eye drop	% found Mean <sup>a</sup> ± SD	Standard addition technique <sup>a</sup>		
		Taken	Added	Recovery%
NPZ	100.29 ± 1.47	20 µg mL <sup>-1</sup>	10 µg mL <sup>-1</sup>	99.20
			20 µg mL <sup>-1</sup>	101.15
			40 µg mL <sup>-1</sup>	98.90
		Mean ± SD		99.75 ± 1.22
PHN	99.52 ± 1.24	50 µg mL <sup>-1</sup>	25 µg mL <sup>-1</sup>	99.80
			50 µg mL <sup>-1</sup>	99.82
			100 µg mL <sup>-1</sup>	101.34
		Mean ± SD		100.32 ± 0.88

<sup>a</sup> Average determinations of four eye drop dosage form solution.

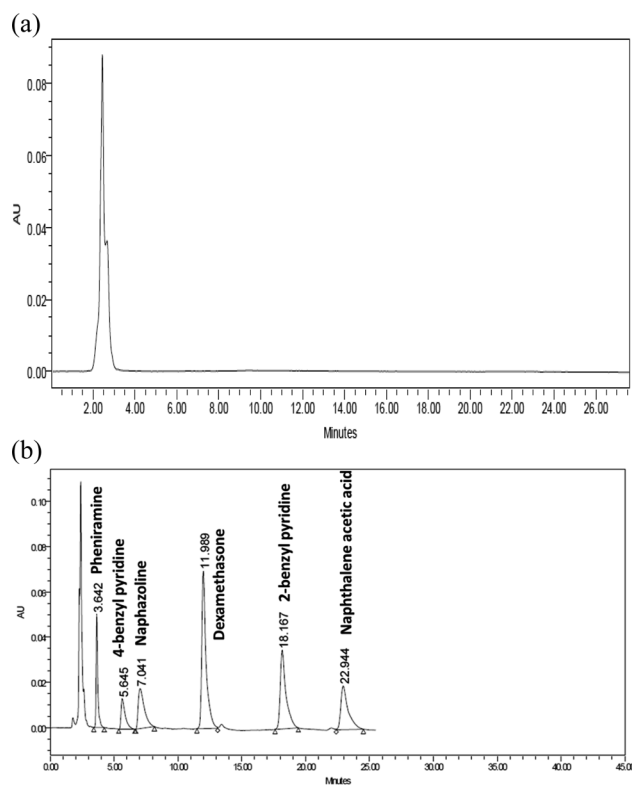
and the aqueous humor spiked with five cited drugs and internal standard. Dexamethasone was chosen as it gives a good resolution with similar retention time with respect to studied components. The  $t_R$  values for aqueous humor spiking were  $3.64 \pm 0.1$ ,  $5.64 \pm 0.1$ ,  $7.04 \pm 0.1$ ,  $11.99 \pm 0.1$ ,  $18.17 \pm 0.1$  and  $22.94 \pm 0.1$  min for PHN, PHN impurity B, NPZ, IS, PHN impurity A and NPZ impurity B respectively. It was found that no peaks corresponding to the retention times of the analyzed components are present in chromatogram of blank aqueous humor (Fig. 3a and b).

**3.5.2. Linearity and sensitivity.** NPZ and NPZ impurity B show their linearity in range of 5.0–45.0 µg mL<sup>-1</sup> where PHN, PHN impurity A and B show their linearity in range of 10.0–110.0, 10.0–70.0 and 10.0–120.0 µg mL<sup>-1</sup>, respectively. The curves were constructed by plotting the peak area ratios (of the analyte to the internal standard) vs. concentrations. The analysis was performed as described before under procedure section. The obtained result of regression equations are summarized in Table 5.

**3.5.3. Recovery.** The recovery was determined by analyzing six QC samples at three different QC level (QCL, QCM and QCH) and comparing them with six plain standards of the same concentrations. Internal standard showed same extraction recoveries at concentration of 100.00 µg mL<sup>-1</sup>. The obtained

recoveries percentages were tabulated in Table 5 indicating that the extraction recoveries are reproducible.

**3.5.4. Precision and accuracy.** Six replicates of QC samples at different four concentration levels (LLOQ, QCL, QCM and QCH)



**Fig. 3** (a) HPLC chromatograms of blank aqueous humor using a hypersil ODS column (5 mm, 250–4.6 mm i.d.), mobile phase of pH 6.0 phosphate buffer: acetonitrile (70 : 30, by volume), flow rate of 1.0 mL min<sup>-1</sup> and detection at 260 nm. (b) HPLC chromatograms of NPZ (45.00 µg mL<sup>-1</sup>), PHN (110.00 µg mL<sup>-1</sup>), three of their selected impurities; NPZ impurity B (45.00 µg mL<sup>-1</sup>), PHN impurity A (70.00 µg mL<sup>-1</sup>), PHN impurity B (120.00 µg mL<sup>-1</sup>) and IS (100.00 µg mL<sup>-1</sup>) in spiked aqueous humor using a hypersil ODS column (5 mm, 250–4.6 mm i.d.), mobile phase of pH 6.0 phosphate buffer: acetonitrile (70 : 30, by volume), flow rate of 1.0 mL min<sup>-1</sup> and detection at 260 nm.

**Table 4** Statistical comparison between the results obtained by the proposed chromatographic HPLC-DAD method and the official BP method of analysis of NPZ, PHN

Parameter	HPLC		Official BP method [10]	
	NPZ	PHN	NPZ	PHN
Mean of recoveries	100.35	99.62	99.63	99.71
SD	0.72	1.15	0.98	1.15
Variance	0.51	1.32	0.96	1.32
N	5	5	5	5
Student's <i>t</i> -test	1.329 (2.306) <sup>a</sup>	0.113 (2.306) <sup>a</sup>	NA	NA
<i>F</i> -test	1.863 (6.388) <sup>a</sup>	1.011 (6.388) <sup>a</sup>	NA	NA

<sup>a</sup> Those values represent the corresponding tabulated values of *t* and *F* at *p* = 0.05.



Table 5 Regression and validation parameters for determination of the studied drugs in rabbit aqueous humor by the proposed HPLC-DAD method

Parameters	PHN	PHN impurity B	NPZ	PHN impurity A	NPZ impurity B
LLOQ ( $\mu\text{g mL}^{-1}$ )	10.00	10.00	5.00	10.00	5.00
<b>Recovery% (QC samples)</b>					
QCL	83.73	88.89	87.14	82.24	84.12
QCM	81.58	88.59	87.34	83.55	85.70
QCH	83.98	87.34	88.19	83.76	84.90
<b>Accuracy (recovery%)</b>					
Inter-run	100.40	99.63	98.94	99.26	98.72
Intra-run	99.17	99.91	99.03	100.01	98.66
<b>Precision</b>					
Inter-run (CV%) <sup>a</sup>	8.98	10.43	9.45	11.43	8.54
Intra-run (CV%)	9.23	13.71	9.83	10.25	12.31
<b>IS - normalized MF (CV%)</b>					
QCL	14.59	11.98	8.89	10.01	13.11
QCH	12.11	11.34	9.78	9.19	12.20
<b>Stability study (recovery%)</b>					
<b>Short - term</b>					
QCL	99.98	99.17	99.38	100.10	99.26
QCH	99.64	99.72	100.15	99.81	99.94
<b>Long-term</b>					
QCL	100.67	99.79	99.45	100.15	99.43
QCH	99.85	99.67	99.35	99.00	98.38
<b>Freeze-thaw</b>					
QCL	99.79	99.57	99.24	98.98	99.38
QCH	99.90	99.89	99.44	99.64	100.16
<b>Dilution integrity (recovery%)</b>					
For 25% level	99.98	100.42	100.77	98.90	99.30
For 50% level	100.34	99.75	99.14	100.61	99.69

<sup>a</sup> CV: coefficient of variation.

were determined by applying the previous chromatographic method. Intra-run precision and accuracy were conducted in three runs while inter-run precision and accuracy were evaluated on three successive days. The precision of the method was expressed as the percent coefficient of variation (% CV). The CV% For the LLOQ should be <20%, and for other concentrations should be <15%. The obtained results were tabulated in Table 5.

**3.5.5. Matrix effect.** Matrix effect was measured at two levels (QCL and QCH) by calculating matrix factor (MF) and IS normalized MF to ensure that the matrix has no significant effect on suppression or enhancement of the efficiency of the drugs extraction. MF was evaluated by dividing the peak area of the analyte in case of matrix presence to the peak area of the analyte in case of matrix absence. Normalized MF was estimated by dividing MF of the analyte to the MF of the IS. The percentage coefficient of variation (CV%) at the two levels (QCL, QCH) was calculated and tabulated in Table 5.

**3.5.6. Stability.** Short- (for 24 h) and long-term (for 30 days at  $-20\text{ }^{\circ}\text{C}$ ) stability of standard solutions spiked with rabbit aqueous humor were checked at two levels of concentrations (QCL and QCH) and recovery percentages were calculated.

Samples were then analyzed by applying the previously mentioned chromatographic conditions. In case of freeze-thaw testing, aliquots of the five studied compounds were individually prepared and subjected to three consecutive cycles of freezing at  $-20\text{ }^{\circ}\text{C}$  overnight and leaving to thaw at room temperature. NO significant changes in the concentrations of the studied drugs after exposing to three cycles were found, indicating that the components are stable during transport process. The results are tabulated in Table 5.

**3.5.7. Dilution integrity.** It was estimated by spiking the matrix with high concentration of the five cited drugs above ULOQ and then diluting the sample to be in the range of the assay (25%, 50% levels). Six samples were prepared and diluted twice and another six were diluted four times to be in the range of assay. Samples were analyzed by the proposed method. The recoveries percent were calculated indicating that the method was accurate and precise for both dilution factors. Results are summarized in Table 5.

### 3.6. Analysis of spiked rabbit aqueous humor samples

The sensitivity of this method allowed the determination of the drugs in biological fluid as rabbit aqueous humor. In spite of



**Table 6** Recovery of NPZ, PHN and their official pharmacopeial impurities NPZ impurity B, PHN impurity A and B from spiked aqueous humor by the proposed HPLC method

Mix <sup>a</sup>	PHN			PHN impurity B			NPZ			PHN impurity A			NPZ impurity B		
	Added conc.	Found conc.	Recovery%	Added conc.	Found conc.	Recovery%	Added conc.	Found conc.	Recovery%	Added conc.	Found conc.	Recovery%	Added conc.	Found conc.	Recovery%
Mix 1	20.00	19.97	99.84	20.00	19.84	99.19	10.00	10.09	100.94	25.00	24.71	98.84	15.00	15.02	100.10
Mix 2	30.00	29.89	99.62	40.00	39.60	98.99	20.00	20.15	100.76	35.00	34.73	99.22	20.00	20.02	100.09
Mix 3	60.00	59.82	99.70	60.00	60.23	100.38	25.00	24.79	99.15	45.00	44.92	99.83	30.00	30.58	101.92
Mix 4	70.00	70.11	100.15	90.00	90.10	100.11	35.00	34.99	99.97	50.00	50.27	100.54	40.00	39.95	99.88
Mix 5	90.00	90.35	100.39	120.00	119.80	99.83	45.00	44.51	98.91	70.00	68.65	98.07	45.00	44.94	99.86
Mean ± SD	99.94 ± 0.32			99.70 ± 0.59			99.95 ± 0.92			99.30 ± 0.94			100.37 ± 0.87		

<sup>a</sup> Average determinations of four laboratory-prepared mixtures.

shifting in retention time occurred, good resolution between peaks was obtained. IS was added to ensure extraction efficiency (Fig. 3b). The values of the obtained recovery percentage are shown in Table 6.

## 4 Conclusion

A chromatographic HPLC-DAD method was developed for the simultaneous determination of naphazoline HCl, pheniramine maleate along with three of their pharmacopeial impurities (naphazoline impurity B, pheniramine impurity A and B). The method was also applied in analysis of the five cited components in biological fluid after spiking rabbit aqueous humor. The proposed method successfully detected trace amounts of official impurities and it could be a good candidate for impurity profiling of the two cited drugs. The method was validated according to ICH guidelines and U.S. FDA bioanalytical validation guidelines for determination of the five cited components in eye drops and spiked aqueous humor, respectively. As a result of the simplicity and successfulness of the method in determining the studied drugs in spiked aqueous humor, it is applicable for quality control laboratories, bioequivalence and bioavailability centers.

## Author contributions

Khadiga M. Kelani: conceptualization, methodology, software, validation, visualization, supervision, project administration, funding acquisition, writing – original draft. Maha A. Hegazy: conceptualization, methodology, software, formal analysis, data curation, visualization, supervision, project administration, funding acquisition, writing – review & editing. Amal M. Hassan: methodology, software, validation, formal analysis, investigation, funding acquisition, project administration, writing – original draft, writing – review & editing. Mahmoud A. Tantawy: methodology, software, validation, formal analysis, investigation, funding acquisition, project administration, writing – original draft, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

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