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# Highly sensitive sequential injection determination of p-aminophenol in paracetamol formulations with 18-molybdodiphosphate heteropoly anion based on elimination of Schlieren effect

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ABSTRACT

A highly sensitive, precise and automated method using sequential injection analysis to assay quantitatively low levels of the p-aminophenol (PAP) in paracetamol formulations has been developed. A solution containing PAP and paracetamol is injected into an acetate buffer carrier stream and merged on-line with 18-molybdodiphosphate heteropoly complex reagent to form a specific blue derivative that is subsequently detected spectrophotometrically at 820 nm. The procedure has been optimized mainly with respect to measurement sensitivity. It is based on the leveling off of the refraction indices of the liquids mixed in the flow system by the careful matching of the refractive index of the reagent solution with that of the carrier and sample solutions. Amount as low as 0.5 $\mu$mol L$^{-1}$ of PAP, which corresponds to the 0.001% of PAP in paracetamol tablets, can be reliably determined using the proposed method, which is clearly below the specification limits recommended for PAP determination in paracetamol drug and tablet formulations (50 ppm or 0.005% (w/w)). The developed method was successfully applied to the analysis of paracetamol formulations spiked with PAP and determination of PAP content in Rapidol tablets past their expiration date both by proposed SIA and reference HPLC methods.

## 1. Introduction

p-Aminophenol (PAP) can exist as either a synthetic intermediate in pharmaceutical preparations or as a primary hydrolytic degradation product of paracetamol. PAP is considered to be an impurity in paracetamol. It is a substance of modest toxicity, able to cause nephrotoxicity and having teratogenic effects. PAP is limited to a low level (50 ppm or 0.005% (w/w)) as a drug substance by Pharmacopoeias in Europe, the United States, Great Britain and Germany using a manual colorimetric limit test. The limit for PAP is broadened to 1000 ppm or 0.1% (w/w) for tablet product monographs, which mention the use of an automated and less sensitive HPLC method. At such a low level pharmacopoeial HPLC assay was not applicable due to matrix interference. A fast, automated assay was necessary for routine analysis. Determination of PAP is frequently used as a step in many methods based on the determination of paracetamol by its hydrolysis to PAP [1,2].

Various methods have been reported for the determination of PAP, including HPLC [3], capillary electrophoresis [4], spectrophotometry [5], fluorimetry [6] and electrochemical techniques [7,8]. Flow methods have been recognized as being potentially more sensitive and faster for the determination of PAP than HPLC analysis and other techniques [9].

FIA/SIA methods are characterized by high sensitivity, which under certain conditions can be significantly higher than that achieved with the relevant batch methods. In order to achieve low limits of detection, special attention should be paid to those factors that determine the signal-to-noise ratio. One such important factor is the Schlieren effect, which limits the sensitivity and affects the signal-to-noise ratio while impairing the reproducibility of spectrophotometric measurements [10].

The Schlieren effect is the result of light inflections caused by the formation of optical artifacts, such as when a mirror or a lens is within the flowing reaction area. The perfect mixing of a sample with reagents and carrier solvents usually cannot be achieved in flow analysis. Gradients of concentrations or sudden changes in local concentrations – that is, a difference in the refraction index along the monitored zones – lead to deflections of light that alter the intensity of the transmitted beam.

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Depending on the mixing conditions [10], the Schlieren effect can consist of two primary components. The first one is associated with the formation of stable liquid lenses – cylindrical layers having different refractive indices – under a laminar flow regime. The lenses increase or decrease the measured signal by focusing light onto or from the detector. The sequence of a positive peak followed by a negative peak, sometimes interchanged in places, is superimposed on the basic recorded output signal, thus distorting it. This component can be reproduced quite easily. The other component appears under poor mixing conditions and leads to the occurrence of a variety of transient mirrors within the flowing sample, thus leading to a noisy recorded signal and a decrease in reproducibility.

Various strategies have been proposed for the elimination of the Schlieren effect, including dual-wavelength treatment of the signal [10], matching the refraction indices of the carrier and sample solutions [11], reversed flow [12], a nested loop [13] and the introduction of large sample volumes [14].

When the dual-wavelength method is employed, the intensities of two selected monochromatic beams are measured simultaneously using separate detectors, and real-time subtraction of the wavelength-independent noise is obtained. The transmitted light is measured at two different wavelengths: one at which the product absorbs light and another outside the product's absorbance spectrum, where only the Schlieren effect is observed. Nevertheless, this methodology requires the generally complex handling of additional data and measurements at another wavelength, which together have the effect of complicating the analysis. The Schlieren effect is wavelength sensitive and noisy, thus leading to both systematic and random errors. To compensate for the Schlieren effect when using this methodology, a diode-array or CCD spectrophotometer is required, and while this method is preferable to analyzing samples having a high concentration of the analyte or other substances, the matching of the refractive index of the carrier with that of the sample and/or reagent solutions is potentially beneficial when highly concentrated carrier or reagent solutions have to be used.

This paper presents a highly sensitive, precise and automated method using Sequential Injection (SI) analysis to assay quantitatively low levels of the p-aminophenol in paracetamol formulations as degradation product. A solution containing PAP and paracetamol is injected into a buffer carrier stream and merged on-line with 18-molybdodiphosphate heteropoly complex (18-MPC) reagent to form a specific blue derivative which is subsequently detected spectrophotometrically at 820 nm. The procedure has been optimized mainly with respect to measurement sensitivity. A new strategy based on the careful matching of the refractive index of the reagent solution with that of the carrier and sample solutions has been proposed to reduce the Schlieren effect that occurs in the flow system used due to high concentrations of acetate buffer and 18-MPC reagent below the required level.

## 2. Materials and methods

### 2.1. Reagents

All reagents used were of analytical-reagent grade and distilled water was used throughout. The p-aminophenol was obtained from Sigma (St Louis, MO, USA). The PAP stock solution was prepared daily by dissolving the appropriate amount of the drug in 0.05 mol $L^{-1}$ HCl solution to reach a final concentration of 0.01 mol $L^{-1}$. This solution was then stored in a refrigerator. Before being used it was diluted to the desired concentration by adding 20 mL of acetate buffer and adjusting the volume to 100 mL with water. The concentrated acetate buffer solution of pH 5.0 ± 0.2 was prepared by dissolving 10.1 g of sodium acetate in 50 mL of water, mixing in a 4.0 mL of glacial acetic acid and adjusting the resulting mixture to a volume of 100 mL. The carrier solution was prepared by using water to dilute 20 mL of concentrated buffer solution to the mark in a 100 mL volumetric flask.

The ammonium salt of the α-isomer of 18-molybdo-2-phosphate heteropolyanion $(NH_4)_6P_2Mo_{18}O_{62} \times 14H_2O$ was synthesized as described in the literature [15]. The 0.01 mol $L^{-1}$ solution of 18-MPC was prepared by dissolving 0.7855 g of the synthesized salt and diluting it to 25 mL with distilled water. If any small insoluble residue appeared, the solution was filtered. The 1 mmol $L^{-1}$ solution of 18-MPC was prepared in a 25 mL volumetric flask by mixing 2.5 mL of the 0.01 mol $L^{-1}$ solution of 18-MPC with 3.0 mL of concentrated buffer solution and diluting to the mark with distilled water. This solution is unstable and should be used within 2 h of preparation.

### 2.2. Sequential injection system

A commercial FIAlab® 3500 system (FIAlab® Instruments, USA) with a syringe pump (syringe reservoir 5 mL) and an 6-port selection Cheminert valve (Valco Instrument Co., USA) was used. A tungsten light source and a USB 2000 UV–VIS fiber optic CCD detector (OceanOptics, USA) were connected to the flow system via 600 μm i.d. optical fibers having SMA connectors (FIAlab® Inc., Bellevue, USA). The entire SIA system was controlled using the latest version of the FIAlab program for Windows. Flow lines were made of 0.75 mm i.d. PTFE tubing. 10, 20 and 50 mm optical Z-flow through cells were used.

### 2.3. HPLC apparatus

The comparative HPLC system, made up of a binary pump LCP 4100 (Ecom, Prague), a Waters 717 plus auto sampler, a Waters 486 Tunable UV detector (Waters, Milford, MA) and a PC for data processing, was controlled by the chromatographic software CSW v.1.7 for Windows (Data Apex s.r.o., Prague). Sample compounds separation was performed on an Onyx Monolithic C18, with a 50 mm × 4.6 mm column (Merck, Germany). The optimal mobile phase for the separation of p-aminophenol and paracetamol was a mixture of acetonitrile:water in a 10:90 (v/v) ratio, with the pH adjusted to 2.8 through the use of orthophosphoric acid (8.5%). Helium was used to degas the mobile phase prior to application.

The final optimized conditions selected were as follows: injection volume of 10 μL for the PAP sample; the isocratic mobile phase was pumped at a flow rate of 1 mL $min^{-1}$ at ambient temperature; and the detection wavelength was 210 nm. A 0.05 mg $mL^{-1}$ solution of caffeine was used as the internal standard.

### 2.4. General SIA procedure

The configuration of the SIA manifold employed for the determination of PAP is shown in Fig. 1. The analytical cycle began by filling the piston pump syringe with 1000 μL of the carrier solution (an acetate buffer solution), which was drawn into the syringe at a flow rate of 50 μL $s^{-1}$. This was followed by 75 μL of reagent (1 mmol $L^{-1}$ M solution of 18-MPC in acetate buffer) and 160 μL sample or PAP standard aspirated sequentially into the holding coil at 30 μL $s^{-1}$ through separate ports (ports no. 2 and no. 3, respectively) of the multi-position valve. The entire volume was then propelled at 30 μL $s^{-1}$ through the Z-flow cell using port no. 6. A spectrometer reference scan was made, and absorbance scanning began immediately.

### 2.5. Determination of PAP in paracetamol formulations

Five paracetamol tablets were accurately weighed and crushed into a powder. An amount equivalent to one tablet (500 mg) was

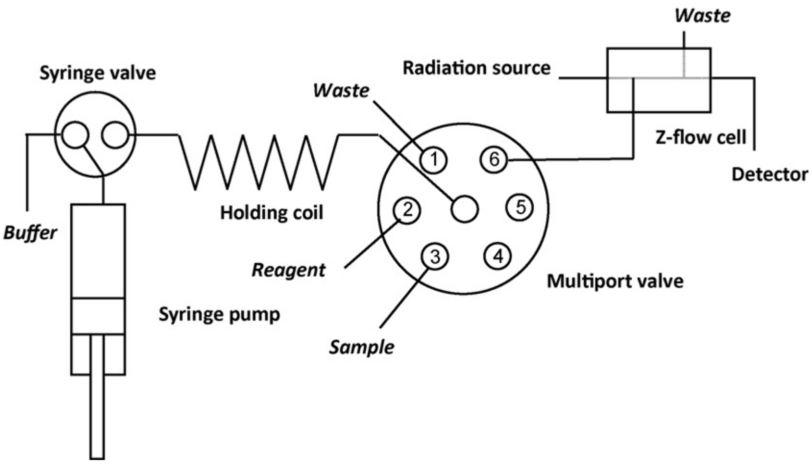

**Fig. 1.** Schematic diagram of the SIA manifold used for PAP determination.

weighed, dissolved in water and transferred to a 100-ml volumetric flask. The mixture was placed into an ultrasonic bath for 5 min, and the volume was filled up. The solution was then centrifuged at 5000 rpm for 15 min and filtered through a 0.45 μm membrane filter. An appropriate aliquot of this solution was used for the analysis.

## 3. Results and discussion

### 3.1. Color reaction of 18-MPC with PAP

18-MPC has several chemical properties which markedly distinguish it among other heteropoly anions used for the determination of reducing agents. These are its comparatively high oxidation potential, its rapid rate of reaction with the reducing agents and the strong coloration of the reduced forms. It can be easily obtained in its pure form.

The influence of the solution pH on the formation of the heteropoly blue produced during the reaction of PAP and 18-MPC was investigated (Fig. 2). At pH > 5.0 PAP is almost immediately oxidized by 18-MPC. In weakly acidic as well as in strongly basic solutions from 4.0 to 11.5 PAP can reduce 18-MPC. An acid medium should be preferred for the reaction, as at pH > 7 the gradual destruction of 18-MPC occurs. Keeping the reaction rate and the stability of heteropolyanion in mind, a pH of 5.0 was chosen as a compromise value. At this pH 18-MPC is reduced almost immediately by PAP. Paracetamol, on the other hand, is capable of reacting with 18-MPC only in a basic medium at pH > 7, and the rate of its reaction is very slow under these conditions. Thus, the determination of PAP is possible in the presence of a large excess of paracetamol.

The spectrum of the heteropoly blue obtained by the reduction of 18-MPC with PAP is identical with that of the 2-e heteropoly blue formed in the reaction between ascorbic acid and 18-MPC [16]. The absorption band maximum for this heteropoly blue at pH 5.0 occurs at 820 nm, with a molar absorptivity of 12,600 $\text{mol}^{-1}\,\text{L}\,\text{cm}^{-1}$. Despite the behavior of ascorbic acid, PAP is not capable of reducing 18-MPC to more deeply reduced heteropoly compounds such as 4-e heteropoly blue. The intersection point on the molar ratio curve is found at a PAP:18-MPC molar ratio of 1:1, confirming the formation of the aforementioned 2-e heteropoly blue and p-benzoquinoneimine considered as main compound produced by oxidation of PAP [17] (Fig. 3).

### 3.2. Overcoming the Schlieren effect in SIA procedure

Because the developed method must be capable of determining small amounts of PAP in the presence of the excess of paracetamol, the sensitivity of the method should be properly adjusted. It was discovered during the development of the method that a significant Schlieren effect influences the determination of PAP at low concentrations. The SIA peaks from the analyte deteriorate, and strong deviations from linearity are observed on the calibration graph at low PAP concentrations.

A comparatively high concentration of 18-MPC should be created in the solution to ensure a broad range of linearity for the

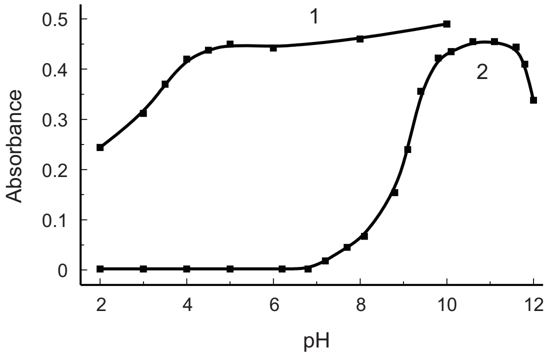

**Fig. 2.** Influence of acidity of the medium on the absorbance of 18-MPC heteropoly blue by reduction with PAP (1) or paracetamol (2). $C_{18\text{-MPC}} = 0.1\ \text{mmol L}^{-1}$, $C_{\text{PAP}} = 0.04\ \text{mmol L}^{-1}$, $C_{\text{paracetamol}} = 0.04\ \text{mmol L}^{-1}$, $\lambda = 820$ nm, $l = 1$ cm.

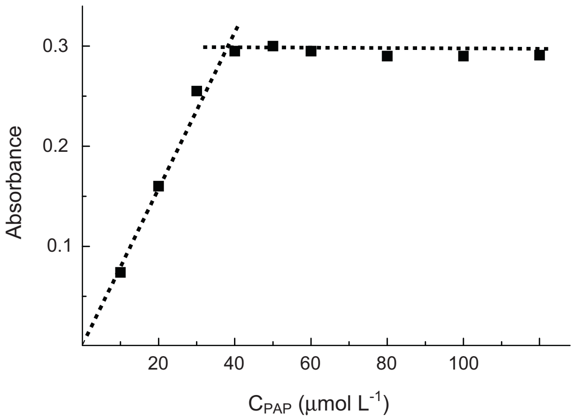

**Fig. 3.** Dependence of the absorbance of the heteropoly blue formed in the reaction between PAP and 18-MPC on PAP concentration. $C_{18\text{-MPC}} = 0.04\ \text{mmol L}^{-1}$, pH = 5, $\lambda = 820$ nm, $l = 1$ cm.

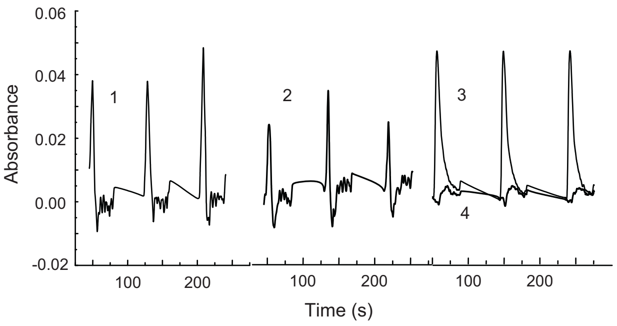

**Fig. 4.** Influence of the Schlieren effect on the SIA signals of 4 $\mu$mol L$^{-1}$ PAP (1, 3) and blank (2, 4) solutions, pH = 5.0, $l$ = 1 cm. Composition of the reagent solution: C(18-MPC) = 0.01 mol L$^{-1}$ (1, 2), C(18-MPC) = 1 mmol L$^{-1}$ and 3.0 mL of conc. acetate buffer in 100 mL of final solution (3, 4).

analyte and the complete formation of the colored product. Taking into account the high molar weight of the ammonium salt of 18-MPC (3142 g mol$^{-1}$), a considerable mass concentration of the heteropoly complex is present in the solution. This may lead to a significant difference in the refractive indices between the mixed liquid zones and consequently to high Schlieren effect.

As can be seen from Fig. 4, at PAP concentrations of less than 4 $\mu$mol L$^{-1}$, the magnitude of the Schlieren effect peak becomes comparable with the signal from the analyte. Thus, the determination of smaller PAP concentrations becomes significantly more complicated. An attempt was made to increase the sensitivity of the method and to reduce the influence of the Schlieren effect on measurements by increasing the length of the flow cell. Under the conditions used in the method the analytical signal is well-scaled. Increasing the flow cell length to 5 cm proportionally increases the height of the PAP peak but simultaneously causes an increase in the positive and especially the negative components of the peak of Schlieren effect (Fig. 5).

In this work, a new strategy has been proposed for removing the Schlieren effect. First of all, the concentration of the 18-MPC reagent was diminished as much as possible. The Schlieren effect was also decreased, yet it remained significant. Secondly, all of the solutions used were prepared using the same final concentration of the buffer solution, including the carrier and sample solutions. Finally, the concentration of the buffer in the reagent solution was adjusted until the Schlieren effect was reduced to the extent possible. During the preparation of the carrier and sample solutions, 5 mL of the initial concentrated acetate buffer solution were added to a 100 mL flask. The optimal concentration of the buffer in the reagent solution was less than in other solutions used, considering the significant contribution of 18-MPC in the Schlieren effect. Under the optimal conditions determined, the maximum value of the peak of Schlieren effect was less than 0.003 absorbance units (both positive and negative components) (Fig. 4), which allowed for the improvement of the method's sensitivity by one order of magnitude.

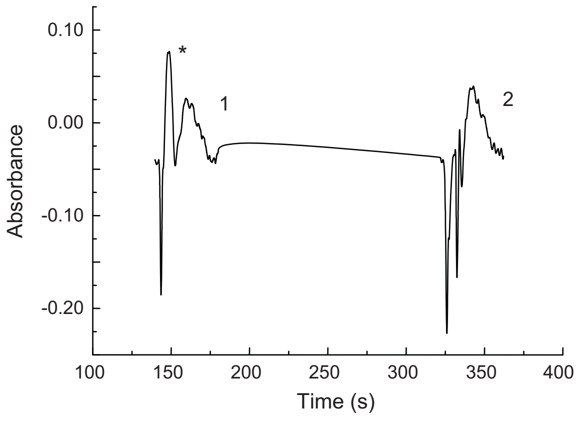

**Fig. 5.** Influence of the Schlieren effect on the SIA signals of 4 $\mu$mol L$^{-1}$ PAP (1) and blank (2) solutions. C(18-MPC) = 0.01 mol L$^{-1}$, $l$ = 5 cm. The PAP peak is marked by an asterisk (*).

The order used for mixing of the reagents in the holding coil in which the concentrated reagent solution is added to the sample solution is much better than the opposite order. The signal obtained was up to 10% greater when a delay time of 120 s was set. However, this extended the time of the analysis too greatly, so this possibility was rejected. The dependence of the absorbance on the injected reagent and sample volumes stopped to grow when these volumes were set at 75 and 160 $\mu$L, respectively.

### 3.3. *Interference study*

At pH 5.0, in contrast to the behavior in the basic medium, the selectivity of the reaction between 18-MPC and reducing agents significantly increases. Only the most active substances interfere with the determination of PAP, including ascorbic acid, cysteine and hydroquinone (Table 1). Catecholamines interfere beginning at a concentration of 0.1 mmol L$^{-1}$. However, even a very large excess of paracetamol (0.1 mol L$^{-1}$) and uric acid (0.02 mol L$^{-1}$) do not interfere, nor do 0.1 mol L$^{-1}$ concentrations of glucose, sacharose and citric acid; 0.012 mol L$^{-1}$ nicotinamide and caffeine; 3 mmol L$^{-1}$ acetylsalicilic acid and common excipients of the pharmaceutical formulations.

### 3.4. *Linearity of the method*

The calibration curve for the SIA determination of PAP was linear in the range of analyte concentrations from 0.5 $\mu$mol L$^{-1}$, which corresponds to the 0.001% of PAP in paracetamol tablets, to 50 $\mu$mol L$^{-1}$. Linear absorbance versus concentration (in mol L$^{-1}$) relationship was obtained with the equation for the calibration graph $A = (1.242 \pm 0.013) \times 10^4\ (b \pm s_b)$ and correlation coefficient of 0.9990 through use of a flow cell of 2 cm path length. The value obtained for the slope is only two times lower than the molar absorptivity coefficient of the 2-e heteropoly blue, which is evidence of good mixing conditions achieved. The intercept was statistically insignificant. Deviations in the calibration curve from the linearity were observed for the PAP concentrations higher than 50 $\mu$mol L$^{-1}$, possibly due to the decreased yield of the colored product. The sample throughput was of 50 h$^{-1}$.

**Table 1**
Effect of foreign ions on the determination of 10 $\mu$mol L$^{-1}$ of PAP.

| Species added | Maximum tolerable molar ratio |
|---|---|
| $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $NO_3{}^-$, $SO_4{}^{2-}$, $PO_4{}^{3-}$, $Cl^-$ | >10,000 |
| $Na_2SO_3$ | 500 |
| $Cu^{2+}$ | 400 |
| $Fe^{2+}$, $Fe^{3+}$ | 60 |
| $NaNO_2$ | 10 |
| Paracetamol | >10,000 |
| Glucose, sacharose, citric acid | >10,000 |
| Uric acid | 2000 |
| Hydrazine sulphate, nicotinamide, caffeine | 1200 |
| Acetylsalicilic acid, thiamine | 300 |
| Phenol | 100 |
| Norepinephrine, methyldopa | 10 |
| Ascorbic acid, cysteine, hydroquinone | 0.1 |

**Table 2**
Recovery data for PAP spiked pharmaceuticals.

| Pharmaceutical formulation[a] | Added ($\mu$g tablet$^{-1}$) | Found ($\mu$g tablet$^{-1}$)[b] | Recovery (%) |
|---|---|---|---|
| Panadol extra | 74 | 74.3 | 100.4 |
| | 148 | 144 | 97.3 |
| | 296 | 287 | 97.0 |
| Paracetamol | 74 | 73.6 | 99.5 |
| | 148 | 146 | 98.5 |
| | 296 | 294 | 99.3 |
| Saridon | 74 | 72.2 | 97.6 |
| | 148 | 151 | 101.8 |
| | 296 | 292 | 98.9 |

[a] Composition of samples: Panadol extra (GlaxoSmithKline): paracetamol 500 mg, caffeine 65 mg; Paracetamol (Ranbaxy): paracetamol 500 mg; Saridon (Bayer): paracetamol 250 mg, propyphenazone 150 mg, caffeine 50 mg.

[b] Average of four determinations.

### 3.5. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection was calculated as three times the ratio of the standard deviation of intercept to the slope of the calibration plot in the low-concentration region (i.e. LOD = 3 × $s_a$/slope) and the limit of quantification as 10 times this ratio (LOQ = 10 × $s_a$/slope). The limit of detection for PAP was found to be 0.18 $\mu$mol L$^{-1}$ (0.02 ppm), and the limit of quantification was 0.5 $\mu$mol L$^{-1}$.

### 3.6. Precision

To assess the repeatability, RSD (%) was calculated for each standard solution used in the preparation of the calibration curve. For the concentration ranges of 10–50, 4–10 and 1–3 $\mu$mol L$^{-1}$, RSD changed in the ranges of 1.0–2.5, 2.5–3.5, and 3.5–8, respectively. For reproducibility, a batch of 500 or 250 mg of ground pharmaceuticals containing paracetamol was spiked with PAP equivalent to 74, 148, and 296 $\mu$g per tablet (Table 2). The mean of all recoveries obtained was 98.22 with a combined RSD of 1.55.

### 3.7. Accuracy

The present method was tested by the determination of PAP in pharmaceutical formulations. The concentration of this degradation product was found to be below the limit of detection in all samples except one, suggesting that the PAP concentration was less than 0.001% the amount of paracetamol. Recovery studies were carried out with the three pharmaceuticals spiked with PAP. Each sample was analyzed four times. As can be seen in Table 2 the recoveries obtained were close to 100%.

The developed SIA as well as HPLC methods were applied to the determination of PAP in Rapidol tablets past their expiration date. The presence of two peaks on the chromatogram proves that PAP was present in significant quantities in the studied pharmaceutical only as a degradation product. In the optimized conditions of the HPLC method, using of the monolithic column was resulted in reduced analysis time and adequate resolution for the PAP and paracetamol (Fig. 6). The data in Table 3 clearly indicate that the PAP content, as measured by the proposed SIA method, was in good agreement with those obtained by the HPLC method.

**Table 3**
Determination of PAP in pharmaceutical formulations using the proposed method and the HPLC reference method (%/tablet ± standard deviation, $n = 5$).

| Pharmaceutical formulation | SIA method | Reference HPLC method | *t*-Test |
|---|---|---|---|
| Rapidol[a] | 0.0120 ± 0.0011 | 0.0134 ± 0.0024 | 1.17 |

[a] Composition of sample: Rapidol (Actavis) 500 mg acetaminophen, inactive ingredients: Eudragit E 100, Eudragit NE 30D, colloidal silica, granulated mannitol, mannitol powder, crospovidone, aspartame, banana essence, magnesium stearate. Production date 04/2008; Expiry date 04/2011; Date of the analysis 07/2011.

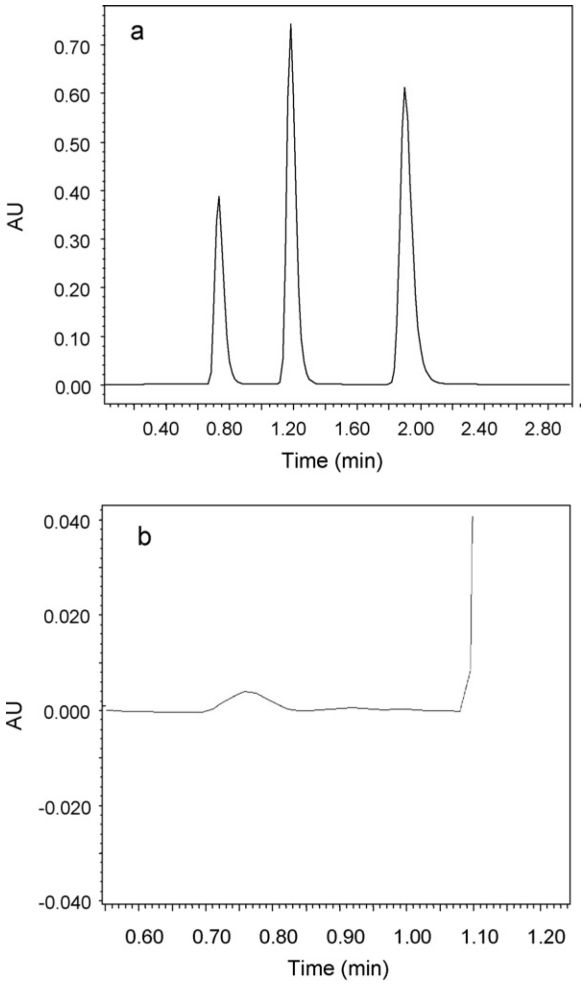

**Fig. 6.** HPLC chromatograms for: (a) standard solution: 50 mg L$^{-1}$ PAP, 100 mg L$^{-1}$ paracetamol, and 50 mg L$^{-1}$ caffeine; (b) Rapidol sample solution. For the experimental conditions see Section 2.3.

The student's *t*-test was used to compare the average contents of PAP in the Rapidol tablets. The calculated value of t (1.17) is less than the critical value of *t* (2.31) at $P = 0.05$ and 8 degrees of freedom, thus showing the absence of significant differences between the average PAP concentrations found by the SIA and HPLC methods.

## 4. Conclusions

A new approach enabling a decrease in the Schlieren effect to below the required level has been proposed. It is based on the leveling off of the refraction indices of the liquids mixed in the flow system by careful matching of the reagent concentration and buffer solution components. The use of this approach to remove the Schlieren effect successfully led to an improvement in the sensitivity of PAP determination with 18-MPC in acetate buffer medium by one order of magnitude. A range of other compounds used as active components of pharmaceutical products and behaving as reducing agents can be determined using the regularities found. Application of the proposed strategy is useful for removing the Schlieren effect in many other FIA/SIA applications.

The developed method was successfully applied to the determination of PAP in paracetamol tablets. Amount as low as 0.5 $\mu$mol L$^{-1}$ of PAP, which corresponds to the 0.001% of PAP in paracetamol tablets, can be reliably determined using the proposed method, which is clearly below the specification limits recommended for PAP determination in paracetamol drug and tablet formulations.

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