Technical paper

Statistical Optimization of Reverse Phase High Performance Liquid Chromatography for the Analysis of Caffeine Paracetamol and its Degradation Product *p*-aminophenol

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Abstract

Paracetamol, analgoantipyretic and anti-inflammatory drug, is available in numerous pharmaceutical formulations in common combination with caffeine and some other drug substances. *p*-aminophenol is paracatamol's process-related impurity that also may be present in formulations containing paracetamol. This paper presents a RP-HPLC method for simultaneous determination of caffeine, paracetamol and p-aminophenol, using chromatographic system Hewlett Packard 1100 series. In defining optimal RP-HPLC chromatographic conditions for the separation of these three compounds, experimental design was applied. Completely separation was achieved using Zorbax Extend C18 column (150 mm × 4.6 mm, 5 μm), with mobile phase consisting of methanol-phosphate buffer pH 6 (20:80 v/v), flow rate of 1 ml/min, and column temperature of 30 °C. UV detection was performed at 230 nm.

Keywords: RP-HPLC, caffeine, paracetamol, *p*-aminophenol, statistical optimization

1. Introduction

Caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione, a central nervous system stimulans, having the effect of temporarily warding off and restoring alertness, are effective for the acute treatment of tension type headache. Paracetamol (*N*-acetyl-*p*-aminophenol) is one of the most popular non-steroidal anti-inflammatory drugs, widely used for the management of pain and fever as major ingredient in numerous cold and flu medications. *Para*-aminophenol (4-aminophenol) is the primary hydrolytic product of paracetamol which has been limited at a very low level in the drug substance by the European and British Pharmacopoeias.[1,2] The low level of *p*-aminophenol are required to ensure paracetamol drug safety, since *p*-aminophenol is reported to have significant nephrotoxicity and teratogenic effects.[3]

So far, a various analytical techniques have been described for determination of paracetamol and caffeine, ei-

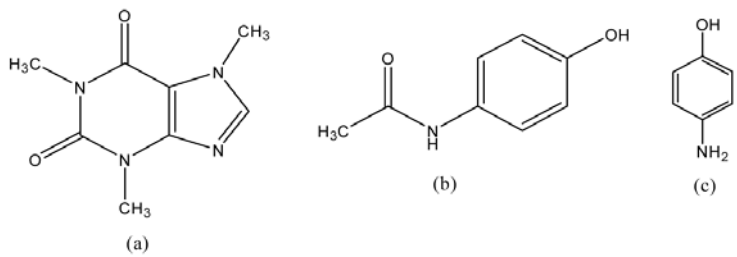

Fig. 1. Structures of caffeine (a), paracetamol (b), p-aminophenol (c)

ther alone or in combination with other drugs.[4–6] However, HPLC is currently most convenient method for simultaneous determination of different compounds frequently associated in formulations against mild pain and common cold. Paracetamol was successfully separated from acetylsalicylic acid, caffeine and phenobarbital in tablets using Bio SiL HL C18, 5 μm, 250 × 4.6 mm column, and mixture of acetonitrile–water (25:75 v/v) adjusted to pH 2.5 with phosphoric acid as a mobile phase.[7]

Separation from caffeine and aspirin in formulations on adsorbents with immobilized nitrile groups using acetonitrile and potassium phosphate in the mobile phase are also achieved.[8]

Simultaneous determination of paracetamol, caffeine, and codeine phosphate on swabs collected from pharmaceutical manufacturing equipment surfaces was described.[9] Paracetamol, caffeine and codeine phosphate were also separated using a μBondapack C_8 column by isocratic elution with mobile phase composition 420/20/30/30 (v/v/v/v) 0.01 M KH_2PO_4, methanol, acetonitrile, isopropyl alcohol.[10] Simultaneous assay of phenylpropanolamine hydrochloride, caffeine, paracetamol, and chlorpheniramine maleate in tablet using HPLC with diode array detection was described.[11]

Considering nephrotoxicity and teratogenic effects of p-aminophenol as impurity, several HPLC methods have been developed to separate and quantify p-aminophenol from multicomponent pharmaceutical formulations. Simultaneous determination of paracetamol, chlorzoxazone and their related impurities, including p-aminophenol, in bulk raw materials and solid dosage forms, with water-methanol-glacial acetic acid (60 + 40 + 2, v/v/v) as mobile phase and Spherisorb ODS 1, 25 cm × 4.6 mm, 5 μm column as stationary phase was described.[12] *p*-aminophenol was separated from paracetamol, phenylephrine and chlorpheniramine in pharmaceutical formulations.[13] A method for the determination of p-aminophenol, by HPLC with amperometric detection was performed in an isocratic mode on a reversed phase Luna column 5 μm C-18 (100 × 4.6 mm) with mobile phase 0.05 mol L^{-1} LiCl solution containing 18% methanol adjusted to pH 4.0 with orthophosphoric acid.[14]

Concerning the numerous of articles dealing with simultaneous determination of paracetamol, caffeine and other analgetic and antipyretic substance, this article is our contribution to analytical community. Thus, here we defined optimal RP-HPLC chromatographic conditions for the separation of paracetamol, caffeine and *p*-aminophenol applying experimental design method.

2. Experimental

2. 1. Material and Reagents

All chemicals were at least analytical grade. HPLC grade methanol was obtained from Lab-Scan, Dublin, Ireland. Phosphoric acid used for adjusting pH of mobile phase was purchased from Carlo Erba, Milano, Italy. Sodium hydrogen-ortophosphate was obtained from Merck, Germany. Deionised water was used throughout. Panadol Extra tablets, each containing 50 mg caffeine and 400 mg paracetamol was manufactured by GlaxoSmith Kline.

2. 2. Apparatus

The HPLC system was used was HP 1100 Hewlett Packard with UV detector. It is consisted of two HP 1100 model G 1316 A pumps, injector Rheodyne, Rheodyne, USA, column thermostat HP 1100 model G 1316 A, sample loop 20μl, Rheodyne, USA. The chromatographic and the integrated data were recorded using an IBM, PC pentium Vectra XA computer system. For processing of data were used two excellent programmes Chem Station and Origin.

2. 3. Chromatographic Conditions

Before the mobile phase was delivered into the system, solvent-A containing 0.01 M sodium hydrogen-ortophosphat (pH adjusted to 6 with orthophosphoric acid) and solvent-B containing methanol, were filtered through 0.45 μm Whatman filter and degassed using ultrasonic bath. Separation of components was performed by using a reversed-phase Zorbax Extend-C18 analytical column 4.6 × 150 mm, 5m particle size. The flow rate was kept constant at 1.0 ml/min at column temperature of 30 °C. The detection was performed at 230 nm.

2. 4. Analytical Procedures

Solutions of caffeine (0.5 mg/ml) and paracetamol (4.0 mg/ml), were prepared in mixture of methanol and water (20:80 v/v). Stock solution of *p*-aminophenol (0.2 mg/ml) was freshly prepared in pure methanol because it is non-stable in presence of water. Stock standard solutions of caffeine and paracetamol are stable on temperature of + 4 °C for 30 days. These solutions were adequately diluted with mixture of methanol and water (20:80 v/v) to determine the accuracy, precision, linearity and limits of detection and quantification. Quantity of powdered Panadol extra tabletes containing 50 mg caffeine and 400 mg paracetamol were transferred into 100 ml volumetric flask, 70 ml mixture of methanol and water (20:80 v/v) was added, mixed in ultrasonic bath for 10 min. and diluted to volume with the mixture methanol/water. This solution was adequately diluted, too.

3. Results and Discussion

3. 1. Optimization of Chromatographic Conditions

During preliminary investigations of chromatographic behaviour of caffeine, paracetamol and *p*-amino-

phenol, the influence of mobile phase composition (% of methanol and pH) and temperature of column were investigated. Capacity, selectivity factor and resolution were chosen as a dependent variable. Preliminary investigations showed selectivity and resolution factor below the critical value and those variables were excluded in further experiments. Because of similar structure of paracetamol and *p*-aminophenol (process-related impurity) and similar retention behaviour, capacity factor for those two substances was very poor as well as separation. Thus, separation under preliminary conditions was not achieved and required further optimization.

For defining optimal conditions full factorial design, for a total of 8 experiments was performed. In full factorial design, the number of experiments corresponds to all possible combinations of selected factors and levels[15] meaning that for great number of factors, for example 8, at two levels, 256 experiments are required.

Full factorial design is most useful when the number of factors is relatively limited. Typical factors in HPLC determinations are, for example, the pH and composition of mobile phase, temperature of column. This experiment was covered with three variables which were considered as possibly influencing the outcome: percentage of methanol (A), pH of mobile phase (B) and temperature of column (C) (Table 1).

Table 1. Considered variables

Factor	(-)	(0)	(+)
A: % of methanol	15	20	30
B: pH of mobile phase	3	6	7,5
C: temp. of column	25	30	40

In order to maximize the information that could be obtained from experimental data different levels of these variables were selected. The design matrix of the eight treatment combinations of low (–) and high (+) levels of factors as well as outcomes of experiments is shown in Table 2.

By applying ANOVA, the statistical significance of each effect was tested by comparing the mean square against an estimate of the experimental error. Deliberate changes did not affect capacity factor of caffeine (capacity factor of caffeine was in proposed limitations ($1 < k < 10$)) and was excluded in further investigations.

The statistical test showed that two effects had p-values (*probability*) less than 0.05 indicating that they were significantly different from zero at 95 % confidence level. The significant factors were found to be % of methanol ($p = 0.0389$ related to k_1 and 0.013 related to k_2) and pH of mobile phase ($p = 0.0182$ related to k_1 and 0.0141 related to k_2). Temperature of column has no statistical significant influence on retention time of investigated compounds and was kept constant at 30 °C. Mutual interactions of these factors were not of great importance ($p < 0.05$) as well.

In order to identify the optimum experimental conditions a response surface diagram was developed. A response surface can simultaneously represent two independent and one dependent variable when a mathematical relationships between variable can be assumed.

The study was done on related influence of percentage of methanol and pH of mobile phase to capacity factor of examined substances. For each of them 16 experiments were performed.

Lack of fit (LOF) test was carried out in order to evaluate which one of suggested model is appropriate for given response. LOF indicates the variation of the data around the fitted model. If a model shows lack of fit, it should not be used to predict the response – a small F value and high p value, grater than 0.1, are good in test (Table 5)

Based on obtained results, coefficients were calculated characterizing the polynomial of second order. For the applied % of methanol /pH of mobile phase (A/B) system the equations of k_1 and k_2 (capacity factor of p-amonophenol and paracetamol) were obtained:

$$k_1 = 0.31 - 0.071\,A + 0.12\,B + 0.019\,A^2 - 0.097\,B^2 - 0.034\,AB \quad (1)$$

$$k_2 = 1.09 - 0.56\,A - 0.055\,B + 0.15\,A^2 - 0.071\,B^2 + 0.046\,AB \quad (2)$$

Table 2. Factorial design

Trial	*Factor level			Response (capacity factor)		
	% of methanol	pH	temp. of column	k_1	k_2	k_3
1	–			–0.14	2.17	9.58
2		–	+	–0.50	1.93	8.88
3	–	+		0.13	1.79	7.03
4	+	–		0.09	0.64	1.63
5	+	+	–	0.29	0.65	1.68
6	+	–	+	0.08	0.54	1.35
7	–	+	+	0.44	1.56	6.48
8	+	+	+	0.26	0.55	1.85

* Low (–) and high (+) levels of the following factors k_1, k_2, k_3-capacity factors of *p*-aminophenol, paracetamol and caffeine respectively

Table 3. Lack of fit tests

Source	Sum of squares	*DF	F value	P	
p-aminophenol					
Linear	0.033	6	3.56	0.1195	Suggested
2FI	0.027	5	3.59	0.1195	
Quadratic	7.435 E-003	3	1.62	0.3187	Suggested
Cubic	6.735–003	1	10.044	0.8441	
paracetamol					
Linear	0.08	6	9.97	0.0216	
**2FI	0.070	5	10.51	0.0204	
Quadratic	0.024	3	6.02	0.578	Suggested
Cubic	3.38 E-003	1	2.54	0.1861	

* DF = degrees of freedom * 2FI = two factorial interaction

Three dimensional graphs in coded factor spaces are presented in Fig.2 and Fig.3.

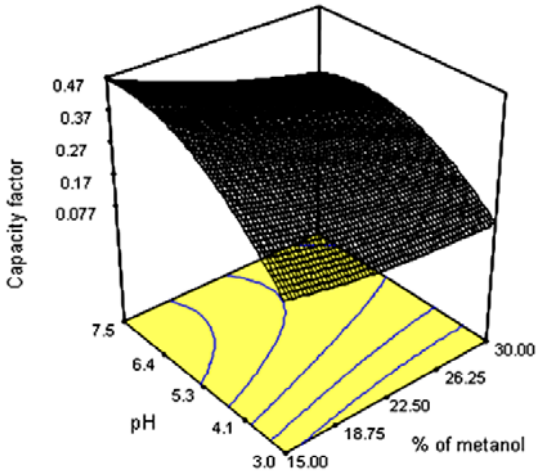

Fig. 2. Three-dimensional graph of k_1 = f (% of methanol /pH of mobile phase) for *p*-aminophenol

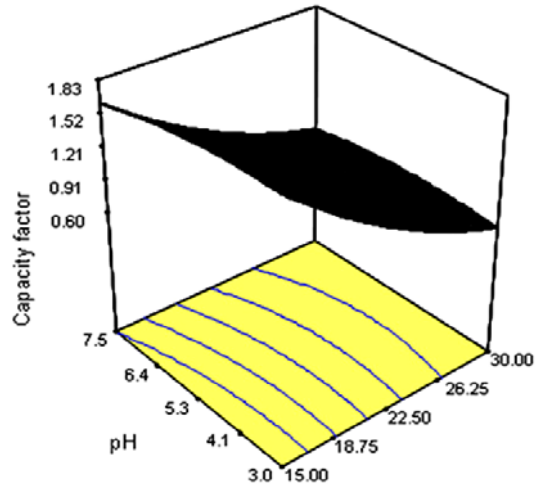

Fig. 3. Three-dimensional graph of k_2 = f (% of methanol /pH of mobile phase) for paracetamol

The negative estimates in the fitted model cause the surface to fold downward quadratically in factor B and increase in factor A. Careful inspection of Fig. 2 and Fig. 3 it was noticed that the "ridge" of the surface is tilted with respect to the factor axes-from the back side toward the front. From the graphs it was concluded that increasing of pH results in increasing of capacity factor of *p*-aminophenol as well as paracetamol.

Moreover, both obtained three-dimensional graph shows acceptable results at pH of mobile phase 7.5 with 15% of methanol. At this condition factor capacity of caffeine is too high, experiment becomes time-consuming and run takes more than 17 min so further experiments were carrying out at pH of mobile phase 6 with 20% of methanol where acceptable results were obtained.

3. 1. 1. Assay

The assay of followed components was estimated using a working standards and the method was validated by the following parameters. Chromatograms of analysis and standards are given on Fig. 4.

Interfering peaks were not detected which indicates good selectivity. The system suitability was confirmed by results: tailing factors were not more than 1.25, resolution was not less than 1.70 and selectivity factor was not less than 1.0. Accuracy of method was checked for all of three examined components; average value of recovery was found to be 99.2% (caffeine), 100.48% (paracetamol), and 99.18% (*p*-aminophenol) and the RSD values are found to be below 1.22%. The precision was studied by repeatability and intermediate precision (the RSD values were found to be below 1.0% for each component at different concentrations).

Linear dependence of the peak area *versus* concentration was determined in the concentration range 0.01–0.1 mg/ml for caffeine, 0.08–0.8 mg/ml for paracetamol and 0.004–0.04 mg/ml for *p*-aminophenol. The regression equations were: y = 103.52x – 22.12, y = 38.28x + 200.80, y = 44.49x + 1.26, for caffeine, paracetamol and p-aminophenol (the coefficient of correlation were 0.999).

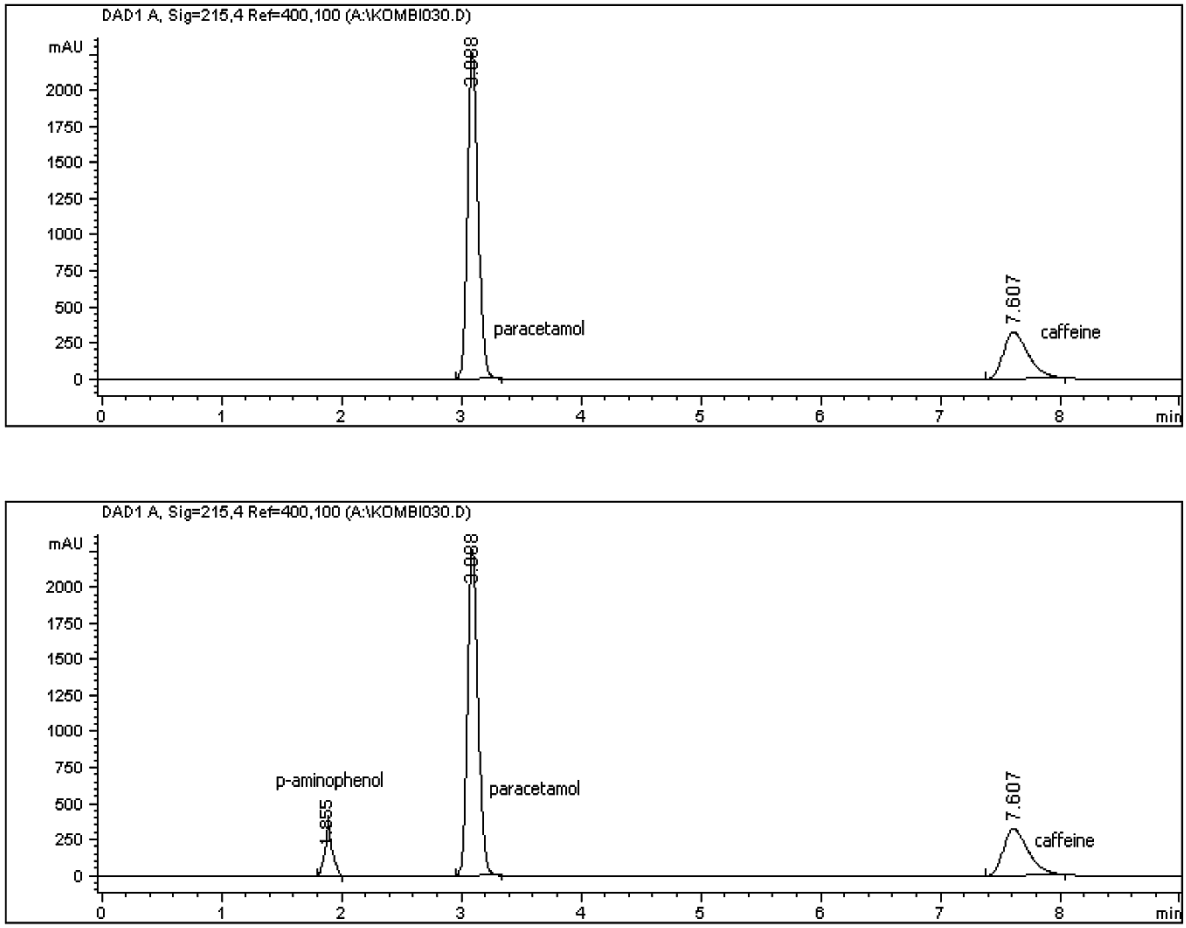

Fig. 4. Chromatograms of analysis and standards of caffeine, paracetamol and *p*-aminophenol. The developed method was validated with respect to specificity, accuracy, precision and linearity.

Limit of detection (LOD) and quantification (LOQ) were determined for impurity (p-aminophenol): 0.023 g/ml and 0.076 g/m respectively.

4. Conclusion

Proposed method is a convenient and efficient method for simultaneous determination of caffeine, paracetamol and p-aminophenol. The developed method does not require using gradient or any procedure of extraction and provides determination (qualitative and quantitative) low levels of the *p*-aminophenol in both paracetamol drug substance and dosage forms. The results obtained in this study corroborate that the proposed HPLC method is sufficiently precise, rapid and sensitive to be used for routine analyses

5. Acknowledgement

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6. References

1. European Pharmacopoeia, 3rd Ed., Council of Europe, Strasbourg **1999**, 748–749
2. British Pharmacopoeia, *Volume II*, HMSO, London **1993**, 483–484
3. L. F. Prescott, in: L. F. Prescott (Ed.): Paracetamol (Acetaminophen) A Critical Bibliographic Review, Taylor & Francis, London, **1996**.
4. C. Akay, T. Degim, A Sayal, A. Aydin, Y. Ozakan, H. Gul, Turk. *J. Med. Sci* **2008**; *38 (2):*167–173
5. R. Nageswara Rao, A. Narasaraju, *Anal. Sci* **2006**, *22*, 287
6. M. Y. Momin, P. G. Yeole, M. P. Puranik, S. J. Wadher, *Indian J. Pharm. Sci* **2006**, *68 (3),* 387–389
7. J. Franeta, D. Agbaba, S. Eric, S. Pavkov, M. Aleksic, S. Vladimirov, *Il Farmaco* **2002**, *57(9),* 709–713
8. G. B. Golubitskii, E. V. Budko, E. M. Basova, V. M. Ivanov, A. V. Kostarnoi, *Zhurnal Analiticheskoi Khimii* **2007**, *Vol. 62*, 636–640

9. A. Schmidt, **2006**, Vol 29(11), 1663–1673
10. M. Kartal, *J. Pharm. Biomed. Anal* **2001**, *26 (5–6),* 857–864
11. G. Indrayanto, A. Sunarto, Y. Adriani, Y, *J. Pharm. Biomed. Anal*. **1995,** *13(12),* 1555–59 *90* (1), 82–93
12. A. M. Shahid, R. Syed, G. Mohsin, K.R Aamer, *J. AOAC International* **2007**, *90 (1),* 82–93
13. A. Marin, E. Gracia, A. Gracia, C. Barbas, *J. Pharm. Biomed. Anal* **2002**, *29,* 701–714
14. E. Wyszecka-Kaszuba E, *J. Pharm. Biomed. Anal* **2003**, *32*, 1081–6
15. T. Lundstedt, E. Seifert, L. Abramo, B. Thelin, A. Nystrom, J. Pettersen, R. Bergman, *Chemometric and Intelligent Laboratory Systems,* **1998,** *42,* 3–40

Povzetek

Paracetamol, analgoantipiretična in antiinflamatorna učinkovina, je na voljo v številnih farmacevtskih pripravkih, običajno v kombinaciji s kofeinom in nekaterimi drugimi učinkovinami. *p*-Aminofenol je običajna nečistoča in predstavlja precesni razgradni produkt paracetamola.

V tem prispevku opisujemo optimizacijo reverznofazne tekočinsko kromatografske metode za hkratno določanje kofeina, paracetamola in *p*-aminofenola z uporabo eksperimentalnega dizajna. Separacijo smo dosegli z uporabo Zorbax Extend C18 kolone (150 mm × 4,6 mm, 5 μm), z mobilno fazo iz metanola in fosfatnega pufra pH 6 (20:80 v/v), pretok 1 ml/min in temperaturo kolone 30 °C. UV detekcijo smo izvedli pri 230 nm.