

Special Article - RP-HPLC

Validated RP-HPLC Method for Quantitative Determination of Tolfenamic Acid and Benzyl Alcohol in a Veterinary Pharmaceutical Preparation

Sebaey Mahgoub^{1,2*}¹Research and Development, Unipharma, Egypt²Food Analysis Laboratory, Ministry of Health, Egypt***Corresponding author:** Sebaey Mahgoub, Research and Development, Unipharma, El-Obour City and Food Analysis Laboratory, Ministry of Health, Zagazig, Egypt**Received:** January 17, 2017; **Accepted:** February 20, 2017; **Published:** February 22, 2017**Abstract**

An isocratic RP-HPLC method coupled with a UV detector was developed and validated for simultaneous assay of tolfenamic acid and benzyl alcohol in a solution for injection formula. Separation was achieved on a Shimadzu Nexera LC-30AD system and was monitored at 220 nm. Elution was carried out at a flow rate of 1 mL/min and isothermally at 40 °C, using acetonitrile and 50mM triethylamine by (70:30, v/v) ratio as the mobile phase on a Hypersil BDS C18 column (4.6 mm x 150 mm, 5 µm). The retention times of benzyl alcohol and tolfenamic acid were 1.87 min and 3.96 min, respectively. The linearity for benzyl alcohol was in the range of 160-240 µg/ml with correlation coefficient of 0.9995 and linearity for tolfenamic acid was in the range of 400-600 µg/ml, with correlation coefficient of 0.9998, respectively. Limit of detection and limit of quantitation were found to be 0.86 and 2.5 µg.mL⁻¹ for benzyl alcohol and 2.1 and 6.7 µg.mL⁻¹ for tolfenamic acid. All validation parameters were within the acceptance range.

Keywords: RP-HPLC; Tolfenamic acid; Benzyl alcohol; Method validation**Introduction**

Tolfenamic acid is a Nonsteroidal Anti-Inflammatory Drug (NSAID) used in the treatment of acute attacks of migraine and the relief of mild-to-moderate pain in disorders such as dysmenorrhea, rheumatoid arthritis or osteoarthritis [1]. It has been introduced as a veterinary treatment and possesses analgesic and antipyretic activities [2]. Tolfenamic acid has an inhibitory action on prostaglandins as effectively as indomethacin and in a considerably lower concentration than acetylsalicylic acid [3]. On the other hand benzyl alcohol is frequently used as an antimicrobial preservative or co-solvent in a variety of pharmaceutical formulations and cosmetic products [4-6] and its concentration should not exceed limit values for each formulation type while it can produce fatal toxic effects, allergies and various other effects on the nervous system [7]. Very high concentrations of benzyl alcohol can result in toxic effects including respiratory failure, vasodilatations, hypotension, convulsions, and paralysis [8]. Several methods have been reported for determination of tolfenamic acid [9-13] and benzyl alcohol [14-21] separately or in presence of different ingredients in Pharmaceutical Preparations. To the best of my knowledge, no HPLC method has been reported yet for assay of tolfenamic acid and benzyl alcohol in a combined formula.

In this paper the first RP-HPLC method for the simultaneous determination of the mixture of tolfenamic acid and benzyl alcohol in a solution for injection formula is reported. The method was able to separate each component as well as other unknown degradation products within a run time of 7 minutes. The study was performed in accordance with established ICH guidelines [22] and was successfully applied for simultaneous determination of the combined formula of these two components.

Experimental Section**Chemicals and reagents**

HPLC-grade acetonitrile, triethylamine and phosphoric acid were manufactured by Fischer Scientific, UK and purchased from their local agent. Standard materials used were working standard available at our laboratories.

Preparation of mobile phase

Buffer solution was prepared by adding 7 mL of triethylamine to 950 mL of purified water, and then pH of the solution was adjusted to 3.0 ± 0.1 with phosphoric acid. The volume was completed to 1000 mL using purified water. Mobile phase consists of a filtered and degassed mixture acetonitrile and 50 mM triethylamine by (70: 30, v/v) ratio.

Chromatographic system

The analysis was performed on a Shimadzu Nexera LC-30AD system (Shimadzu Corporation, Kyoto, Japan) consisting of LC-30 AD-LPGE pump, SPD-20A UV detector and DGU-20A5 Degasser. A Shimadzu SIL-30AC auto sampler was used to inject 1 µL of the samples on Hypersil BDS C18 column (4.6 mm x 150 mm, 5 µm), Thermo Scientific, USA), which was kept at 40°C. The detection was carried out at 220 nm. Mobile phase was filtered through a 0.22 µm membrane filter (Chromatech, UK or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 1.0 mL/min.

Solutions preparation

Standard solution: Standard solution was prepared by dissolving standard substances (working standard) in mobile phase, sonicated for 5 min and diluted up to the mark with mobile phase to obtain a solution containing, 200 µg/ml of tolfenamic acid and 500 µg/ml of benzyl alcohol.

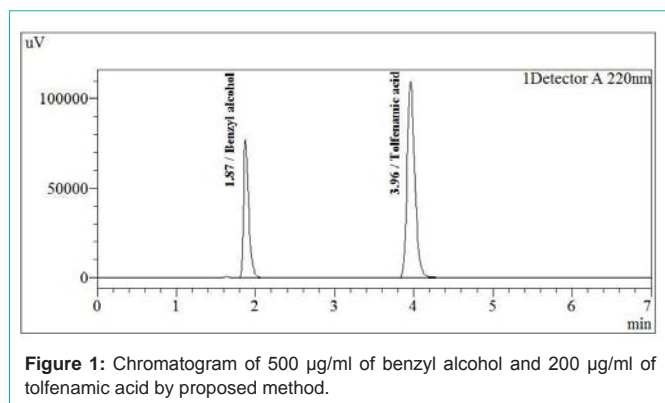


Figure 1: Chromatogram of 500 µg/ml of benzyl alcohol and 200 µg/ml of tolfenamic acid by proposed method.

Sample solution: An accurately measured volume of the formula equivalent to 20 mg of tolfenamic acid and 50 mg of benzyl alcohol were taken in 50 ml volumetric flask. About 30 ml mobile phase was added to this flask and the flask was sonicated in an ultrasonic bath for 5 min and the volume was completed to 50 mL using mobile phase. 5 mL of the previous solution was diluted to 10 mL with mobile phase then filtered through a 0.22 µ syringe filter.

Results and Discussion

Method development and optimization

The main target of the chromatographic method was achieving a method for simultaneous assay for tolfenamic acid and benzyl alcohol. The combined standard solution (200 µg/ml of tolfenamic acid and 500 µg/ml of benzyl alcohol) was injected. For method optimization, the mobile phase ratio, stationary phase and UV wavelength were adjusted to achieve the best retention time and peak resolution between the two analytes. After many trials the method was applied using a Hypersil BDS C18 column (4.6 mm x 150 mm, 5µ) and mobile phase consisting of acetonitrile and 50 mM triethylamine by (70:30, v/v) ratio, giving good separation, acceptable retention time, peak shape, plate's count and good resolution. Chromatogram obtained for the combination upon using final HPLC conditions is shown in (Figure 1).

Method validation

System suitability: System suitability was performed on six replicate injections of mixed standard solution. The Relative Standard Deviation (RSD) values, tailing factor and number of theoretical plates were the chromatographic parameters selected for the system suitability test. The following requirements were fulfilled, % RSD of peak responses due to each component for the six replicate injections must be less than or equal to 1.0 %, tailing factor must be less than 2.0 %, resolution must be more than 2 and theoretical plates count must be more than 2000. System suitability parameters were calculated and are presented in (Table 1).

Linearity: Linearity of any analytical procedure is its ability to obtain results which are directly proportional to concentration of the analytes in the sample. It was studied by preparing standard solution of five concentration levels ranging from 80 % to 120 % of test concentration (160-240µg/ml of tolfenamic acid and 400-600µg/ml of benzyl alcohol) and analyzed in triplicate per level, (Figure 2). The graphs were plotted using peak responses of each component on

Table 1: System suitability parameters (n=6).

No.	Parameter	Benzyl alcohol	Tolfenamic acid
1.	Retention time	1.87	3.96
2.	USP resolution	--	12.965
3.	Tailing factor	1.615	1.237
4.	No. of theoretical plates	3141	7071

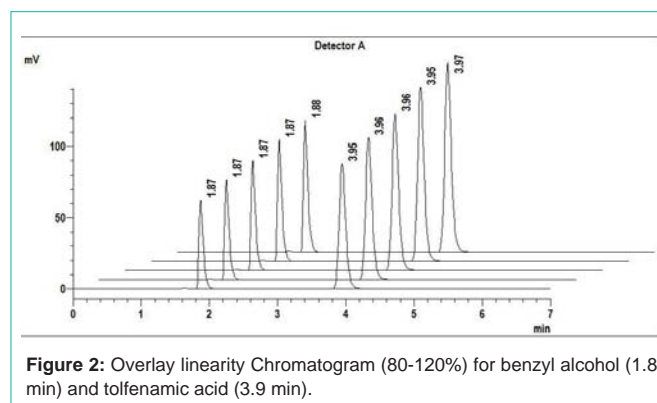


Figure 2: Overlay linearity Chromatogram (80-120%) for benzyl alcohol (1.8 min) and tolfenamic acid (3.9 min).

Table 2: Method precision results.

No.	Tolfenamic acid	Benzylalcohol
1.	751646	351923
2.	750662	351051
3.	752294	352729
4.	751542	351678
5.	752493	351634
6.	751678	351698
Mean	751719.2	351785.5
SD	645.8	546.03
RSD%	0.086	0.155

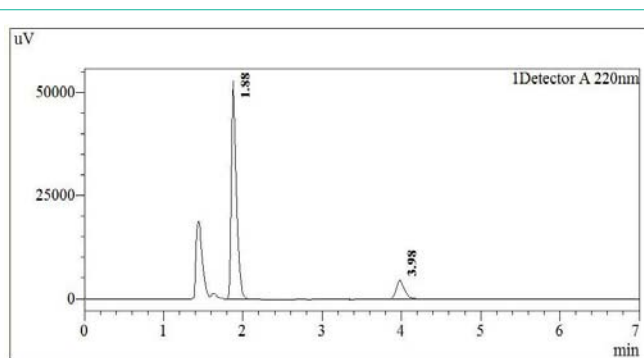
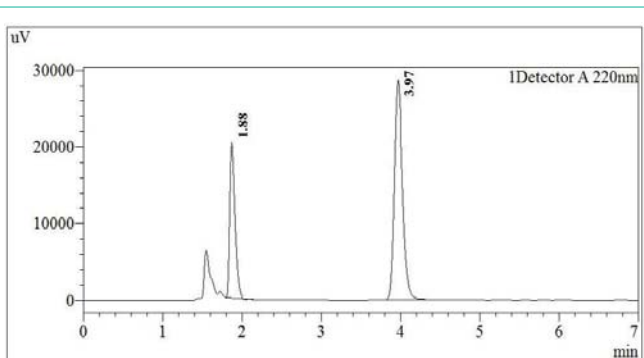
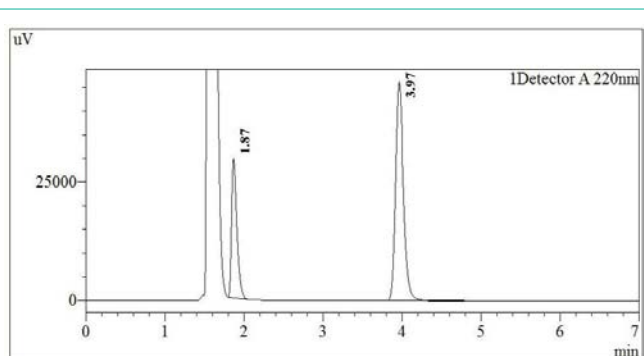
Table 3: Intermediate precision of the method.

Day	Determination	Tolfenamic acid (%)	Benzyl alcohol (%)
1 st	1	99.98	100.10
	2	100.23	100.55
	3	98.84	99.04
2 nd	1	100.26	100.19
	2	99.81	99.55
	3	99.63	98.91
Mean		99.79	99.72
SD		0.52	0.66
%RSD		0.53	0.67

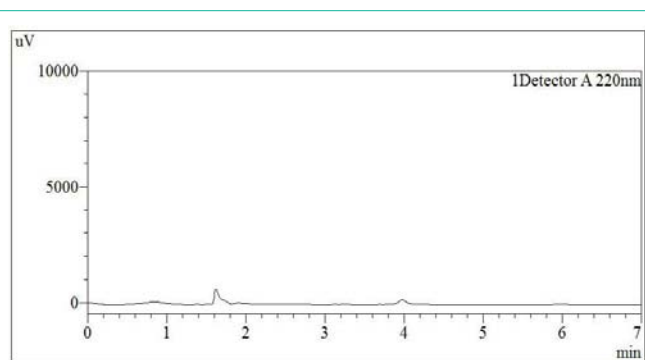
Y-axis and the corresponding concentration on x-axis. The peak area ratios of tolfenamic acid and benzyl alcohol were linear with respect to their concentrations. A good correlation was found between the observed peak area ratios (y) and the theoretical concentration (x). Least-squares regression analysis provided typical regression lines: $y = 3818.4x - 12326$ ($r^2 = 0.9995$) for tolfenamic acid and $y = 677.82x + 12529$ ($r^2 = 0.9998$) for benzyl alcohol.

Table 4: Results of the accuracy study.

Component	Spiked level (% n=3)	Spiked conc. ($\mu\text{g/ml}$)	Recovery (%)
Tolfenamic acid	80	160	99.98
	100	200	100.23
	120	240	100.54
Benzylalcohol	80	400	100.10
	100	500	100.55
	120	600	100.06

**Figure 3a:** Acid degradation chromatogram.**Figure 3b:** Base degradation chromatogram.**Figure 3c:** Oxidizer degradation chromatogram.

Precision: The precision of the method was established by carrying out the analysis of 100% concentration of the analytes for six times. The Relative Standard Deviation (RSD) was used as the measurement of precision. The obtained low value of standard

**Figure 3d:** Placebo chromatogram.

deviation showed that the method is precise. The results obtained are presented in (Table 2). Intermediate precision of the method was examined by comparing the assays performed on two different days, each carried out three determinations of the same test using the described method. The results are given in (Table 3).

Accuracy: The accuracy of the method was verified by adding different amounts of known standards to the sample solution then determining the percentage recovery of each concentration with respect to real values. The accuracy of the assay method was evaluated in triplicate at three concentration levels 80%, 100% and 120% of the label claim. Amount of drug recovered was quantified and % recovery was calculated. The results of the accuracy study are reported in (Table 4). For the two drugs, at the different concentration levels, good recoveries were obtained meeting the acceptance criteria of 100 \pm 2%.

Specificity: Specificity is the ability of the method to measure the analytes response in the presence of its potential impurities [23]. Forced degradation studies were performed to demonstrate selectivity of the method using 0.1M HCl, 0.1M NaOH and 0.1M H_2O_2 . The degradation samples were prepared as 200 $\mu\text{g/ml}$ of tolfenamic acid and 500 $\mu\text{g/ml}$ of benzyl alcohol.

After the degradation process, samples were allowed to cool at room temperature and diluted to the same concentration as that of the standard solution. (Figures 3a-3d) show that there is no interference at the retention time of tolfenamic acid and benzyl alcohol due to placebo or degradation products, indicating that the method is selective.

Robustness: Robustness of the developed method was determined by analyzing the samples after some small but deliberate changes in the method parameters such as change in flow rate (± 0.2 mL/min), change in pH (± 0.2 unit) and change in organic composition of mobile phase by ($\pm 5\%$). Changes in chromatographic parameters such as theoretical plates count and tailing factor were evaluated for the studies and are shown in (Table 5). The method is robust for all tested parameters.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): Sensitivity of the method was proved by establishing the Limits of Detection (LOD) and Quantitation (LOQ) for tolfenamic acid and benzyl alcohol in pure and in dosage form. The LOD and LOQ were determined by visual inspection using signal to noise approach, by

Table 5: Robustness evaluation of the method.

Chromatographic changes	%RSD (Peak Area) n=6	Tailing factor	Theoretical plate count
Tolfenamic acid			
Flow rate (ml/min)			
0.8	0.085	1.237	7101
1.0	0.086	1.236	7053
1.2	0.089	1.238	7069
pH value			
2.8	0.084	1.231	7133
3	0.086	1.234	7055
3.2	0.085	1.236	7070
Organic composition (%)			
65	0.093	1.240	7101
70	0.081	1.241	7071
75	0.090	1.220	7113
Benzyl alcohol			
Flow rate (ml/min)			
0.8	0.157	1.616	3125
1.0	0.155	1.612	3135
1.2	0.160	1.618	3299
pH value			
2.8	0.158	1.614	3288
3	0.154	1.615	3199
3.2	0.156	1.609	3285
Organic composition (%)			
65	0.151	1.614	3265
70	0.159	1.615	3141
75	0.165	1.621	3199

injecting a series of dilute solutions with known concentrations. The concentration (in µg/mL) with signal to noise ratio of at least 3 was taken as LOD and concentration with signal to noise ratio of at least 10 was taken as LOQ. The limit of detection of tolfenamic acid and benzyl alcohol was 0.86 and 2.1 µg/mL, respectively. The limit of quantification was 2.5 and 6.7 µg/mL, respectively.

Conclusion

A simple RP-HPLC method was successfully developed and validated for simultaneous determination tolfenamic acid and benzyl alcohol in a solution for injection formula. All drugs were well resolved in a run time of 7 min. No interference was found with the degradants or excipients. Validation results have proved that the method is selective, precise, accurate and robust and can be successfully applied for the routine analysis.

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References

- Abdelwahab NS, Ali NW, Zaki MM, Abdelkawy M. Validated chromatographic methods for simultaneous determination of tolfenamic acid and its major impurities. *Journal of Chromatographic Science*. 2015; 481-491.
- Niopas I, Georgarakis M. Determination of tolfenamic acid in human plasma by HPLC. *Journal of Liquid Chromatography*. 1995; 18: 2675-2682.
- Niopas I, Georgarakis M. Rapid assay for the determination of tolfenamic acid in pharmaceutical preparations and biological fluids by high-performance liquid chromatography. *Journal of Liquid Chromatography*. 1991; 14: 2951-2967.
- Rizk M, Ibrahim F, Hefnawy M, Nasr JJ. Micellar liquid chromatographic analysis of benzyl alcohol and benzaldehyde in injectable formulations. *Acta Pharmaceutica*. 2007; 57: 231-239.
- Menon GN, Norris BJ. Simultaneous determination of hydroxyzine hydrochloride and benzyl alcohol in injection solutions by high-performance liquid chromatography. *Journal of Pharmaceutical Sciences*. 1981; 70: 697-698.
- Rego A, Nelson B. Simultaneous determination of hydrocortisone and benzyl alcohol in pharmaceutical formulations by reversed-phase high-pressure liquid chromatography. *Journal of Pharmaceutical Sciences*. 1982; 71: 1219-1223.
- Khan KM, Khan AR, Perveen S, Zia-Ullah, Ambreen N, Voelter W. High performance liquid chromatographic quantitative determination of amcinonide and benzyl alcohol in pharmaceutical preparations. *The Open Analytical Chemistry Journal*. 2008; 2: 74-77.
- Khade V, Mirgane S. High performance liquid chromatography method for the

- analysis of benzyl alcohol. *International Journal of Scientific & Engineering Research*. 2014; 5: 887-889.
9. Tsiliou S, Kefala LA, Perdih F, Turel I, Kessissoglou DP, Psomas G. Cobalt(II) complexes with non-steroidal anti-inflammatory drug tolfenamic acid: Structure and biological evaluation. *European Journal of Medicinal Chemistry*. 2012; 48: 132-142.
 10. Gallo P, Fabbrocino S, Dowling G, Salini M, Fiori M, Perretta G, et al. Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A*. 2010; 1217: 2832-2839.
 11. Ibrahim H, Boyer A, Bouajila J, Couderc F, Nepveu F. Determination of non-steroidal anti-inflammatory drugs in pharmaceuticals and human serum by dual-mode gradient HPLC and fluorescence detection. *Journal of Chromatography B: Analytical in the Biomedical and Life Sciences*. 2007; 857: 59-66.
 12. Mikami E, Goto T, Ohno T, Matsumoto H, Inagaki K, Ishihara H, et al. Simultaneous analysis of anthranilic acid derivatives in pharmaceuticals and human urine by high-performance liquid chromatography with isocratic elution. *Journal of Chromatography B: Biomedical Sciences and Applications*. 2000; 744: 81-89.
 13. Shinozuka T, Takei S, Kuroda N, Kurihara K, Yanagida J. Micro determination of anthracitic acid derivatives of anti-inflammatory drugs by high performance liquid chromatography and its application to forensic chemistry. *Eisei Kagaku*. 1991; 37: 461-466.
 14. De Micalizzi YC, Pappano NB, Debattista NB. First and second order derivative spectrophotometric determination of benzyl alcohol and diclofenac in pharmaceutical forms. *Talanta*. 1998; 47: 525-530.
 15. Floora BJ, Klein E, Muhammad N, Ross D. Stability-indicating liquid chromatographic determination of etoposide and benzyl alcohol in injectable formulations. *Journal of Pharmaceutical Sciences*. 1985; 74: 197-200.
 16. Ghasemi J, Niazia A, Ghobadi S. Simultaneous spectrophotometric determination of benzyl alcohol and diclofenac in pharmaceutical formulations by chemometrics method. *Journal of the Chinese Chemical Society*. 2005; 52: 1049-1054.
 17. Hewala I, El-Fatate H, Emam E, Mubrouk M. Development and application of a validated stability indicating HPLC method for simultaneous determination of granisetron hydrochloride, benzyl alcohol and their main degradation products in parenteral dosage forms. *Talanta*. 2010; 82: 184-195.
 18. Perez-Lozano P, Garcia-Montoya E, Orriols A, Minarro M, Tico JR, Sune-Negre JM. A new validated method for the simultaneous determination of benzocaine, propylparaben and benzyl alcohol in a bioadhesive gel by HPLC. *Journal of Pharmaceutical and Biomedical Analysis*. 2005; 39: 920-927.
 19. Shabir GA. Method development and validation of preservatives determination (benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate) using HPLC. *Journal of Liquid Chromatography & Related Technologies*. 2007; 30: 1951-1962.
 20. Cudina OA, Comor MI, Jankovic IA. Simultaneous determination of bifonazole and benzyl alcohol in pharmaceutical formulations by reverse-phase HPLC. *Chromatographia*. 2005; 61: 415-418.
 21. Wood GC, Iyer MR, Geller AM, Fleischner AM, Sheth BB. A high pressure liquid chromatography assay method for analysis of deslorelin and benzyl alcohol in deslorelin injection. *Journal of Liquid Chromatography & Related Technologies*. 1998; 21: 2183-2190.
 22. International Conference on Harmonization (ICH) Q1A (R2): Stability Testing of New Drug Substances and Products, USA: ICH. 2003.
 23. International Conference on Harmonization (ICH) Q2 (R1): Validation of Analytical Procedures-Test and Methodology, Geneva, Switzerland: ICH. 2005.