HPLC Method for the Analysis of Paracetamol, Caffeine and Dipyrone

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An accurate, simple, reproducible and sensitive method for the determination of paracetamol, caffeine and dipyrone was developed and validated. Paracetamol, caffeine and dipyrone were separated using a μ-Bondapack C_8 column by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase composition was 0.01 M KH_2PO_{4-}-methanol-acetonitrile-isopropyl alcohol (420: 20: 30: 30) (v/v/v/v) and spectrophotometric detection was carried out at 215 nm. The linear range of determination for paracetamol, caffeine and dipyrone were 0.409-400 μg/ml, 0.151-200 μg/ml and 0.233-600 μg/ml, respectively. The method was shown to be linear, reproducible, specific, sensitive and rugged.

Key Words: Paracetamol, Acetaminophen, Caffeine, Dipyrone, High performance liquid chromatography, Validation.

Introduction

Paracetamol (acetaminophen) is one of the most popular over-the-counter analgesic and antipyretic drugs. Paracetamol is available in different dosage forms: tablet, capsules, drops, elixirs, suspensions and suppositories. Dosage forms of paracetamol and its combinations with other drugs have been listed in various pharmacopoeias[1,2].

The combination of paracetamol with dipyrone is used as an antipyretic, analgesic and antiinflammatory drug.

Numerous methods have been reported for the analysis of paracetamol and its combinations in pharmaceuticals or in biological fluids. Paracetamol has been determined in combination with other drugs using titrimetry[3,4], voltammetry[5], fluorimetry[6], colorimetry[6], UV-spectrophotometry[7−9], quantitative thin-layer chromatography (TLC)[10], high-performance liquid chromatography (HPLC)[11−16] and gas chromatography (GC)[17] in pharmaceutical preparations.

Dipyrone is an analgesic, antipyretic, and antiinflammatory drug. After oral administration it is hydrolyzed to 4-methylaminoantipyrine and further metabolized to 4-aminoantipyrine, 4-formylaminoantipyrine, and 4-acetylaminoantipyrine[18].

Dipyrone (noramidopyrine methanesulfonate sodium, metamizole) is a water-soluble pyrazolone derivative drug available in oral and parenteral forms. For more than 60 years it has been used as an analgesic,

antipyretic, antispasmodic, and antiinflammatory drug. Although the drug is widely used in many countries, in others it has been restricted or banned because of the alleged risks of adverse reactions, in particular agranulocytosis[18].

Dipyrone has been determined by titrimetric[19], UV-spectrophotometric[8,9,20], colorimetric[21], polarographic[22], TLC, HPTLC[20], gas liquid chromatography (GLC)[23] and HPLC[24] methods.

The simultaneous determination of paracetamol and dipyrone in tablets by derivative spectrophotometry was reported in the literature[25].

Although there are UV[9] and PLS (Partial Least-squares Spectrophotometric)[26] methods for the determination of paracetamol, dipyrone and caffeine in their ternary mixture. A suitable HPLC method to determine the ternary mixture of paracetamol, dipyrone and caffeine was not located in the literature survey.

The objective of this study was to develop and validate a specific, accurate, precise and reproducible quality control method for paracetamol, caffeine and dipyrone in their ternary combination.

Experimental

Chemicals

Paracetamol (acetaminophen) was used from USP reference standard (103-90-2), caffeine was obtained from Merck Chemicals (Merck-2584) and dipyrone was received from United Pharmaceutical Works. Chromatographic gradedouble-distilled water, analytical grade KH_2PO_4 (Merck-104871), HPLC-grade acetonitrile (Merck-100030), methanol (Riedel-de Haen-34860) and isopropyl alcohol (Carlo Erba reagenti-415154) were used.

Apparatus

The method development was performed with a LC system consisting of a Waters model 515 solvent delivery system, a Waters model 996 Photodiode-array detector (MILFORD, MA, USA) and a Waters 717 plus autosampler using a 20-μl sample loop. The system was controlled and data analyses were performed with the Millennium 2010 software. The assays were performed with another LC system consisting of a Jasco model PU- 980 pump and JASCO UV- 975 UV/VIS detector. Samples were injected with a 7725 Rheodyne injector system with a 20 μl sample loop. The detector was set at 215 nm (0.02 a.u.f.s) and peak areas were integrated automatically by computer using the Borwin software program.

Separation was carried out at ambient temperature using a μ-Bondapak C_8 column (5 μm, 250 mmx 4.6 mm I.D.; Waters, Milford, MA, USA). A guard column (10 μm Bondapak C_{18} in disposable plastic inserts and Waters Guard- Pak holder) was used to safeguard the analytical column. All the calculations concerning the quantitative analysis were performed with external standardization by the measurement of peak areas.

Stock and Standard Solutions

Paracetamol (20.00 mg), caffeine (10.00 mg) and dipyrone (15.00 mg) were accurately weighed in a 10 ml volumetric flask and dissolved in the mobile phase and filled up to volume with the mobile phase.

Standard Working Solution

Standard working solutions were prepared individually in mobile phase for paracetamol, caffeine and dipyrone. Aliquots from each working solution were combined and diluted with mobile phase to yield a solution with final concentrations of 100 μg/ml, 50 μg/ml and 150 μg/ml. Studies on the stability of the analytes in standard working solution showed that there were no decomposition products in the chromatogram or difference in areas during analytical procedure, even after storage for four days at $+$ 4 °C.

Procedure

Chromatographic Conditions

HPLC analysis was performed by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase composition was 0.01 M KH_2PO_4-methanol-acetonitrile-isopropyl alcohol (420: 20: 30: 30) (v/v/v/v). All solvents were filtered through a 0.45 μm millipore filter before use and degassed in an ultrasonic bath. Volumes of 10 μl prepared solutions and samples were injected into the column. Quantification was effected by measuring at 215 nm as established from the three-dimensional chromatogram. The chromatographic run time was 10 min and the column void volume was 1.735 min.

Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R), the selectivity (α) and peak asymmetry (T).

Calibration

Mixed standard solutions containing paracetamol (25-400 μg/ml), caffeine (12.5-200 μg/ml), and dipyrone (37.5-600 μg/ml) were prepared in the mobile phase.

Triplicate 10 μl injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The five concentrations of each compound were subjected to regression analysis to calculate the calibration equation and correlation coefficients.

Results and Discussion

Method Development

The mobile phase was chosen after several trials with methanol, isopropyl alcohol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of 0.01 M KH_2PO_4-methanol-acetonitrile-isopropyl alcohol (420: 20: 30: 30) (v/v/v/v) was selected to achieve maximum separation and sensitivity.

Flow rates between 0.5 and 1.5/min were studied. A flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed-phase C_8 column, the retention times for paracetamol, caffeine and dipyrone were observed to be 4.880 min, 5.845 min and 8.093 min respectively. Total time of analysis was less than 9 min.

The maximum absorption of paracetamol, caffeine and dipyrone together as detected at 215 nm and this wavelength was chosen for the analysis.

The chromatogram at 215 nm showed a complete resolution of all peaks (Figure).
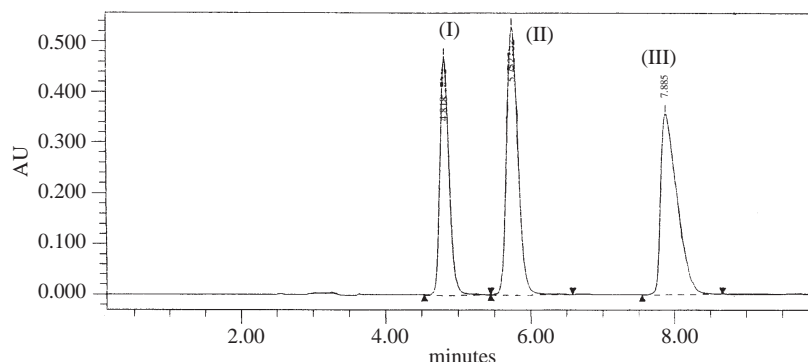
Figure Chromatogram of the mixture of paracetamol **(I)**, caffeine **(II)** and dipyrone **(III)** developed by the LC method

Linearity

Table 1 presents the equation of the regression line, correlation coefficient (r^2), relative standard deviation (RSD) values of the slope and intercept for each compound. Excellent linearity was obtained for compounds between the peak areas and concentrations of 25- 400 μg/ml with r^2 = 0.9987, 12.5- 200 μg/ml with r^2 = 0.9999 and 37.5- 600 μg/ml with r^2 = 0.9993 for paracetamol, caffeine and dipyrone, respectively.

Table 1. Linearity Results, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	λ	Equation	r^2	Slope (RSD%)	Intercept (RSD%)	LOQ μg/mL	LOD μg/mL
Paracetamol	215	**Y**= 20491.74 **X** + 150586.1	0.9987	0.192	2.031	1.226	0.409
Caffeine	215	**Y**= 53717.01 **X** + 197602.2	0.9999	0.150	1.868	0.502	0.151
Dipyrone	215	**Y**= 19043.82 **X** + 111528.5	0.9993	0.058	2.914	0.776	0.233

X= Concentration (μg/ mL); **Y**= Area

Limits of Detection and Quantification

Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at a signal-to-noise ratio (S/N) of 9. LOD and LOQ were experimentally verified by six injections of paracetamol, caffeine and dipyrone at the LOD and LOQ concentrations. The LOD was calculated to be 0.409, 0.151 and 0.233 μg/ml and the LOQ was calculated to be 1.226, 0.502 and 0.776 μg/ml for paracetamol, caffeine and dipyrone, respectively (Table 1).

Suitability of the Method

The chromatographic parameters such as resolution, selectivity and peak asymmetry were satisfactory for these compounds (Table 2). The calculated resolution values between each peak-pair were no less than 3.60 and the selectivity was not less than 1.30.

k′ values were found to be 1.81, 2.37 and 3.66 for paracetamol, caffeine and dipyrone, respectively.

Table 2. System Performance Parameters of Paracetamol, Caffeine and Dipyrone

Compound	t $_r$ (n= 9, mean)	Area (n= 9, mean)	k′	R	α	T
Paracetamol	4.880 (0.33)*	2308090.67 (0.56)*	1.81	3.628 (0.64)*	1.307 (0.24)*	1.179
Cafeine	5.845 (0.47)*	2853026.33 (0.47)*	2.37			1.154
Dipyrone	8.093 (0.47)*	3062225.89 (0.46)*	3.66	6.433 (0.77)*	1.547 (0.16)*	1.505

*RSD% values are given in parentheses

RSD%= (Standard Deviation/Mean) x 100

Precision

The precision of the method (within-day variations of replicate determinations) was checked by injecting paracetamol, caffeine, dipyrone 9 times at the LOQ level. The precision of the method, expressed as the RSD % at the LOQ level, was 2.02, 2.51 and 5.54% for paracetamol, caffeine and dipyrone, respectively (Table 3).

Table 3. Precision of the Developed Method at the LOQ level (n=9)

Compound	λ	Peak Area (mean)	RSD%
Paracetamol	215	24179.44	2.02
Caffeine	215	25541.22	2.51
Dipyrone	215	18617.50	5.54

Accuracy

A standard working solution containing paracetamol, caffeine and dipyrone, yielding final concentrations of 100 μg/ml, 50 μg/ml, and 150 μg/ml respectively was prepared. The prepared mixture of standards was injected 9 times as a test sample. From the respective area counts, the concentrations of the paracetamol, caffeine and dipyrone were calculated using the detector responses. The accuracy, defined in terms of % deviation of the calculated concentrations from the actual concentrations, is listed in Table 4.

Table 4. Accuracy of the Developed Method (n= 9)

Compound	Spiked Concentration μg/mL	Measured Concentration μg/mL Mean ± SD	RSD %	Deviation %
Paracetamol	100	105.28 ± 0.630	0.600	5.28
Caffeine	50	49.43 ± 0.249	0.504	1.14
Dipyrone	150	154.94 ± 0.742	0.479	3.29

$$\% \text{ Deviation} = \frac{\text{(Spiked Concentration - Mean Measured Concentration)}}{\text{Spiked Concentration}} \times 100$$

Ruggedness

The ruggedness of the HPLC method was evaluated by carrying out the analysis using a standard working solution, the same chromatographic system and the same column on different days. The prepared mixture of standards was injected 9 times as a test sample. Small differences in areas and good constancy in retention times were observed after 96 hours. An RSD of less than 0.560% for areas and 0.472% for retention times were obtained (Tables 5 and 6). The comparable detector responses obtained on different days indicate that the method is capable of producing results with high precision on different days.

Similarly, the ruggedness of the method was tested by injecting the standard working solution into a different HPLC unit. The high degrees of reproducibility of detector responses and retention times indicate that the method is fairly rugged.

Table 5. Day to Day Variability According to Area [a]

	June 16, 2000			June 20, 2000		
	Paracetamol	Caffeine	Dipyrone	Paracetamol	Caffeine	Dipyrone
Area	2308090.67	2853026.33	3062225.89	2381848	2949559.88	3020215.89
S.D.	12921.17	13356.87	14124.23	11432.76	9166.10	7476.78
RSD%	0.560	0.468	0.461	0.480	0.311	0.248

[a]Mean values of nine determinations

Table 6. Day to Day Variability According to Retention Time [a]

	June 16, 2000			June 20, 2000		
	Paracetamol	Caffeine	Dipyrone	Paracetamol	Caffeine	Dipyrone
R_t	4.880	5.845	8.093	5.122	6.456	8.934
S.D.	0.016	0.027	0.038	0.007	0.011	0.020
RSD%	0.329	0.468	0.472	0.139	0.171	0.221

[a]Mean values of nine determinations

Analysis of Synthetic Mixtures

Recovery studies in this method were performed on the synthetic mixtures prepared by adding accurately weighed amounts of the drugs (Table 7). Mean recoveries and RSD were found to be 101.62 and 4.28% for paracetamol, 100.11 and 3.38% for caffeine 100.86 and 2.88% for dipyrone, respectively.

Table 7. Recovery Results for Paracetamol, Caffeine and Dipyrone in Synthetic Mixtures by HPLC

Mixture	Paracetamol			Caffeine			Dipyrone		
No	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery
	µg	µg	%	µg	µg	%	µg	µg	%
1	400	400.97	100.24	200	194.20	97.10	600	600.02	100.00
2	300	302.77	100.92	150	155.87	103.91	450	455.34	101.19
3	200	190.84	95.42	100	103.55	103.55	300	289.07	96.36
4	100	105.29	105.29	50	49.43	98.87	150	154.94	103.29
5	50	53.12	106.25	25	24.28	97.12	75	77.60	103.46
			X=101.62			X=100.11			X=100.86
			RSD%=4.28			RSD%=3.38			RSD%2.88

X= Mean

Conclusion

The developed method is suitable for the identification and quantification of the ternary combination of paracetamol, caffeine and dipyrone.

A high percentage of recovery shows that the method can be successfully used on a routine basis.

The proposed method is simple, sensitive, rapid, specific and could be applied for quality and stability monitoring of paracetamol, caffeine and dipyrone combinations.

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