ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2015; 1(12): 187-190
www.allresearchjournal.com
Received: 19-09-2015
Accepted: 20-10-2015

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Method development and validation of Simultaneous Determination of Salicin and Caffeine in Herbal Formulation

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Abstract
A rapid and simple HPLC method has been developed for the simultaneous quantification of Salicin and Caffeine in herbal formulation. Analysis was performed using C_{18} column (250 x 4.6 mm) by isocratic elution with water (containing 0.5% *ortho* phosphoric acid): acetonitrile (90:10) and detection at 213 nm using Ultra Violet (UV) detector. The calibration plot was linear over the range studied (Salicin: 0.5-3.0 µg; Caffeine: 1.0-6.0 µg) with a correlation of 1.0 for both Salicin and Caffeine. The method was also validated for the linearity, range, precision, recovery and detection limits. Thus, the method is suitable for routine analysis of Salicin and Caffeine in herbal formulation.

Keywords: Salicin, Caffeine, HPLC, Analgesic.

Introduction
Pain is defined as unpleasant sensation usually evoked by an external or internal noxious stimulation. An analgesic (also known as a painkiller) is any member of the diverse group of drugs used to relieve pain (achieve analgesia) [1]. In the history of medicine for the relief of pain, many medicinal herbs have been used for their potential therapeutic value. Taking into account that the most important analgesic prototypes (Salicylic acid and morphine) were originally derived from plant sources, the study of plants species traditionally used as pain killers should still be seen as a fruitful research strategy in the search of new analgesic drugs [2]. Early human used to seek remedies from any materials due to the problem of uncontrolled pain. Herbal drugs are considered to be as effective as synthetic drugs with lesser side effects. Herbal medicines are in line with nature, with less hazardous reaction [3].
White willow bark (*Salix alba*) has been widely used as an analgesic. It has been used to treat many different kinds of pain, including rheumatic pain, back pain, toothache, headache, and menstrual cramps. It is also used to relieve sore throat, fever and headache associated with upper respiratory tract infections and influenza [4]. Willow's active chemical constituent, Salicin, was identified in 1829 by the French pharmacist H. Leroux. Salicin was widely used by 19th century European physicians to treat rheumatic fever and as an antipyretic, gout remedy and analgesic, particularly for joint pain [5].
The effect of Caffeine on pain is of interest for several reasons. Caffeine is present in analgesic formulations and exhibits adjuvant properties, increasing the analgesic effect of the primary constituent [6].

Materials and Methods
Chemicals and reagents
All the solvents were of HPLC grade purchased from MERCK India. Water purified by the Milli Q water purification system was used. The standards Salicin and Caffeine were purchased from Sigma Aldrich (India).

Chromatographic Conditions
Shimadzu LC-2010C_{HT} quaternary HPLC system was used having auto injector and UV detector. The LC Solutions software was used for integration. Separation was achieved using

a Phenomenex Gemini C_{18} column (250 x 4.6 mm, 5µ ID). The solvent system consisted of water (containing 0.5% *ortho* phosphoric acid): acetonitrile (90:10) was pumped isocratically at a flow rate of 1.5 ml/min. The detection was carried out at 213 nm by UV detector.

Preparation of standard stock solution

The stock solutions containing 100µg/ml of Salicin and Caffeine were prepared in water. Aliquots of Salicin (0.5-3 µg) and Caffeine (1.0-6.0 µg) were prepared in the mobile phase.

Preparation of sample solution

The components, Salicin and Caffeine from herbal formulation were extracted by water. 10 tablets were taken and pulverized using mortar and pestle. Pulverized sample was transferred quantitatively in a 150 ml conical flask. 90 ml water was added and the solution was sonicated for 15 minutes followed by warming on steam bath for 15 minutes. The solution was allowed to cool. The solution was then filtered into a 100 ml volumetric flask using a Whatman filter paper No 1 and finally the volume was made up with water.

Method Validation

Linearity

The calibration curve was linear over the concentration range of 0.5-3.0 µg for Salicin and 1.0-6.0 µg for Caffeine.

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of Salicin and Caffeine in sample were confirmed by comparing the retention time.

Precision

Three replicated injections at five different concentration of Salicin (0.5, 1.0, 1.5, 2.0 and 3.0 µg) and Caffeine (1.0, 2.0, 3.0, 4.0 and 6.0 µg) were made and analyzed to examine the precision of the method.

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at three levels by adding a known quantity of Salicin and Caffeine to the pre analyzed samples and the mixtures were analyzed according to the proposed method.

Sensitivity

The sensitivity of measurement of Salicin and Caffeine by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were determined by injecting progressively low concentration of solutions. The limit of detection is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio 3). The limit of quantitation is the smallest concentration of the analyte which gives a response that can be accurately quantified (signal to noise ratio 10).

Results and Discussion

Method Development

The HPLC condition was developed and optimized using trial and error method. Various proportions of different solvents as mobile phase with varying flow rates were tried to get resolution of both the compounds. The optimized mobile phase was water (containing 0.5% *ortho* phosphoric acid): acetonitrile (90:10) pumped isocratically at a flow rate of 1.5 ml/min. The optimized mobile phase could resolve both the compounds apart from each other and the peaks obtained were compact too. The detection was carried out at 213 nm by UV detector.

The optimized chromatographic condition yielded a symmetrical peak for both substances with Retention Time (RT) 5.35 minutes (for Salicin) and 10.06 minutes (for Caffeine). The HPLC chromatogram of Salicin and Caffeine is shown in Figure 1.

The developed method was then validated and successfully applied for quantitation of Salicin and Caffeine from the samples. Regression analysis data is shown in Table 1.

The calibration curve of Salicin and Caffeine were linear in the range of 0.5 to 3.0 and 1.0 to 6.0 µg respectively. Precision, expressed in terms of % RSD, analyzing the substances at five different concentrations, in triplicate, summarized in Table 2.

The specificity of the method was assessed evaluating retention times of triplicated injections of five different concentrations of Salicin and Caffeine as standards and in sample. The results were summarized in Table 3, 4 and 5.

To ensure the accuracy of the method, recovery studies were performed by standard addition method at three different levels, to the pre-analyzed samples and the subsequent solutions were re-analyzed. At each level, three determinations were performed and the results obtained are shown in Table 6.

The sensitivity of measurement of Salicin and Caffeine by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were given in Table 7.

The method was applied to the formulation and the chromatogram of the formulation. When the formulation was analyzed in HPLC, Salicin and Caffeine gave sharp and well defined peaks at specific RT. The chromatogram is shown in Figure 2.

Conclusion

In this proposed method the linearity was observed in the concentration range of 0.5 – 3.0 µg for Salicin and 1.0-6.0 µg for Caffeine with co-efficient of correlation, $r^2 = 1.00$ and $r^2 = 1.00$ for Salicin and Caffeine respectively at 213 nm. The result of the analysis of combined mixture by the method was found to be highly reproducible and reliable. The matrix and other ingredients present in the product did not interfere with determination of Salicin and Caffeine. So, the developed HPLC method is simple, precise and accurate and can be used for simultaneous determination of Salicin and Caffeine in herbal formulations.

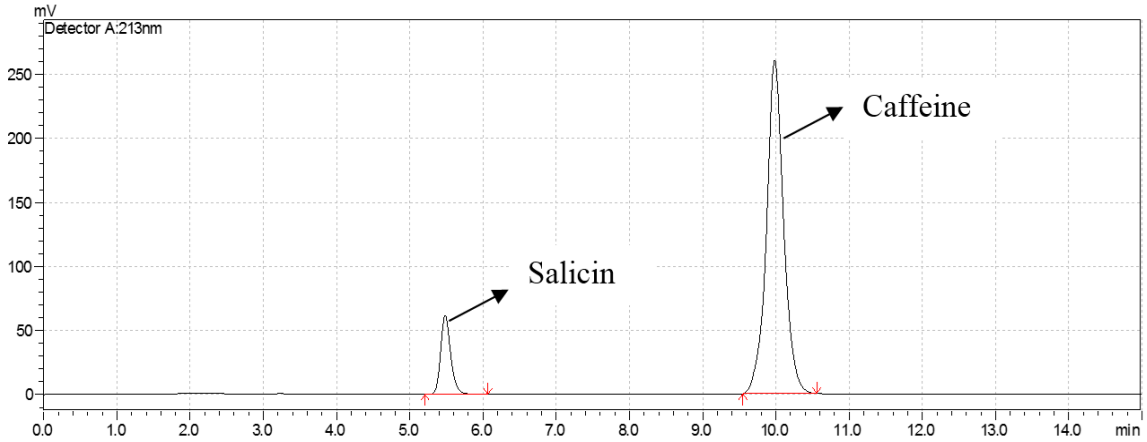

Fig 1: A Typical Chromatogram of Salicin and Caffeine

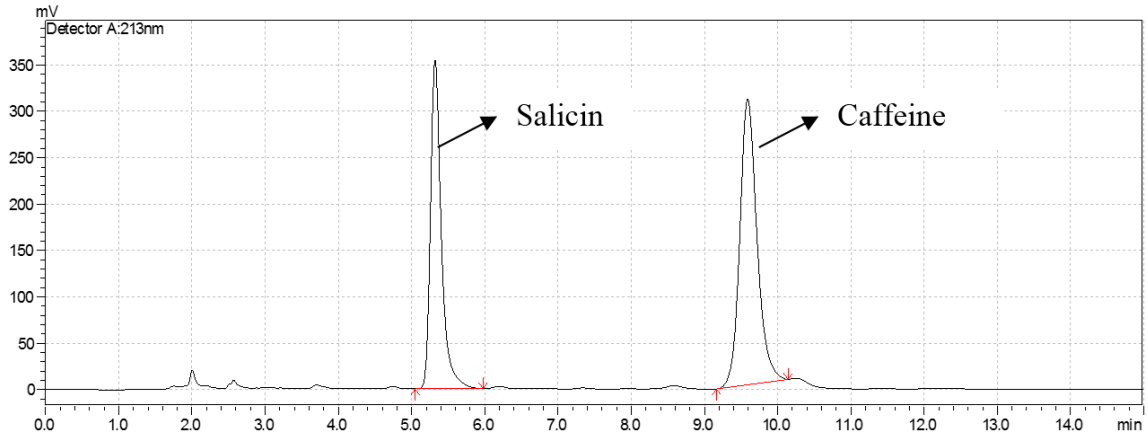

Fig 2: A Typical Chromatogram of Herbal formulation

Table 1: Statistical Data of Salicin and Caffeine

Parameters	Results	
	Salicin	Caffeine
Linearity range (µg)	0.5-3.0	1.0-6.0
Slope	1133255.73	3839786.30
S. D of Slope	113.17	27405.54
Intercept	34990.43	472738.65
S. D. Intercept	772.18	624.82
Regression equation	Y= 1133255.73x+34990.43	Y= 3839786.30x+472738.65
Correlation coefficient	1.00	1.00

Table 2: Precision Data for Salicin and Caffeine

Salicin			Caffeine		
Content in µg	Mean peak area (n=3) ± SD	% RSD	Content in µg	Mean peak area (n=3) ± SD	% RSD
0.5	588394 ± 434.11	0.07	1.0	4291059± 2218.7	0.05
1.0	1167323± 707.19	0.06	2.0	8042643± 10023.5	0.12
1.5	1739276± 2983.36	0.17	3.0	1209677± 5879.7	0.05
2.0	2329806± 262.82	0.01	4.0	15947866± 2318.7	0.01
3.0	3416528± 385.26	0.01	6.0	23421929± 5159.0	0.02

Table 3: Specificity of Salicin standard

Salicin					
Content in µg	0.5	1.0	1.5	2.0	3.0
1	5.483	5.473	5.485	5.489	5.491
2	5.479	5.472	5.49	5.49	5.492
3	5.473	5.476	5.49	5.489	5.491
Mean	5.478	5.473	5.488	5.489	5.491
Stddev	0.005	0.002	0.003	0.0006	0.00057
% RSD	0.092	0.038	0.052	0.010	0.0105

Table 4: Specificity of Caffeine standard

Caffeine					
Content in µg	**1.0**	**2.0**	**3.0**	**4.0**	**6.0**
1	9.989	9.96	9.997	10.002	10.002
2	9.98	9.959	10.004	10.003	10.005
3	9.963	9.968	10.006	10.003	10.004
Mean	9.977	9.962	10.002	10.003	10.003
Stddev	0.013	0.005	0.005	0.001	0.002
% RSD	0.132	0.049	0.047	0.006	0.015

Table 5: Specificity of Salicin and Caffeine in Sample

S No.	Sample A		Sample B	
	Salicin	**Caffeine**	**Salicin**	**Caffeine**
1	5.356	9.835	5.506	10.063
2	5.356	9.835	5.505	10.061
3	5.358	9.838	5.506	10.063
Mean	5.356	9.836	5.505	10.062
Stddev	0.001	0.002	0.006	0.002
% RSD	0.021	0.017	0.010	0.011

Table 6: Recovery data for determination of Salicin and Caffeine

Salicin			Caffeine		
Amount present in sample (µg ± %RSD)	**Amount added [µg]**	**% Recovery ± %RSD**	**Amount present in sample (µg ± %RSD)**	**Amount added [µg]**	**% Recovery ± %RSD**
1.49 ±0.15	1.19	100.98 ± 0.08	1.26 ±0.11	1.02	100.05 ± 0.11
	1.49	109.82 ± 0.11		1.26	103.59 ± 0.33
	1.79	91.94 ± 1.40		1.45	91.57 ± 0.06

Table 7: Results of sensitivity data for Salicin and Caffeine

Parameters	Results	
	Salicin	**Caffeine**
LOD (µg)	1.5	1.2
LOQ (µg)	4.3	3.5

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