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Article

Determination of Glucosamine and Carisoprodol in Pharmaceutical Formulations by LC with Pre-Column Derivatization and UV Detection

Ghada M. Hadad[1]*, Randa A. Abdel-Salam[1] and Samy Emara[2]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, University of Suez Canal, Ismailia 41522, Egypt, and [2]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Misr International University, Egypt

*Author to whom correspondence should be addressed. Email: ghhadad@hotmail.com

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A simple and reliable precolumn derivatization liquid chromatography method with ultraviolet detection has been developed and validated for the analysis of glucosamine (GS) in various dietary supplement formulations and raw materials. Additionally, the proposed method was used for analysis of carisoprodol (CR) found in ternary mixture with paracetamol (PR) and caffeine (CF). The linearity ranges were 1–100 μg/mL for GS, 1–150 μg/mL for CR, PR and CF. Derivatization was used with 1,2-naphthoquinone-4-sulphonic acid sodium salt in the presence of borate buffer. Chromatographic separation of GS-naphthoquinone derivative was achieved by using a mixture of acetonitrile and water (pH 7.3 adjusted with 0.1 M NaOH) in the ratio 10:90, v/v and flow-rate of 1.0 mL/min. UV detection was carried out at 280 nm. For PR, CF, and CR-naphthoquinone derivative, the chromatographic separation was achieved by using mixture of acetonitrile and 20 mM KH_2PO_4 (pH 3.0 adjusted with phosphoric acid) in the ratio 20:80, v/v and flow-rate of 1.0 mL/min. UV detection was carried out at 275 nm. The limits of detection were 37.2, 35.9, 30.4 and 40.0 ng/mL for GS, CR, PR and CF, respectively.

Introduction

The necessity to ensure the quality of pharmaceutical polydrugs and consequently, the safety and efficacy of the final marketed products, has led to the development and evaluation of new methods that can reduce the time and cost of analysis.

Ultraviolet (UV) detection methods in high-performance liquid chromatography (HPLC), which is the most widely used method, sometimes lack sensitivity or selectivity because many substances of interest cannot be detected because they do not contain the necessary chromophoric, fluorophoric or redox groups for trace level drug analysis. Chemical derivatization can modify drugs to give efficient absorption in UV or visible wavelength.

Both glucosamine (GS) and carisoprodol (CR) have no peculiar chromophore; therefore, derivatization for the purpose of adding a strong UV chromophore to the compound is a must for its analysis. From that point, 1,2-naphthoquinone-4-sulphonic acid sodium salt (NQS), which is the color labeling reagent for primary amines in alkaline medium (1) was used as the precolumn derivatizing agent for GS and CR.

GS (2-amino-2-deoxy-d-glucose), an amino monosaccharide, is a natural component found in connective tissues and gastrointestinal mucosal membranes used for the management of osteoarthritis; they may be combined with other substances supposed to be of benefit, including chondroitin, vitamins and various herbs (2).

Several derivatization techniques were accomplished prior to GS analysis using HPLC (3–9) and gas chromatography (GC) (10).

The proposed method for determination of GS has several advantages over the published methods because it is a simple procedure (4) with a short derivatization time and a short run time (5–9). Furthermore, most of the published HPLC methods used for GS derivative separation are based on gradient elution (3, 5–9). Gradient elution has certain drawbacks, such as baseline drift and long run time; however, the proposed method uses isocratic elution.

CR, a skeletal muscle relaxant, is combined with paracetamol (PR), and caffeine (CF) used in the treatment of neuromuscular and joint disorders.

A review of literature revealed that no analytical methods have been reported for the simultaneous determination of PR, CF and CR in their ternary combination. Several methods in the literature describe the simultaneous determination of PR and CF, including liquid chromatography (LC) (11–16), capillary electrophoresis (17), spectrophotometry (18) and high performance thin layer chromatography (18).

The objective of this work was to develop, optimize and validate a simple, rapid and reliable isocratic reversed-phase (RP)-HPLC method with UV detection for determination of GS and CR in their pharmaceutical dosage forms. Conditions affecting the efficiency and reproducibility of the derivatization process for nonchromophoric compounds (GS and CR) were identified and optimized.

Experimental

Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μL loop and a SPD-10AVP UV-VIS detector, separation and quantitation were made on a 250 × 4.6 mm (i.d.) 5 μm C_{18} column (Luna, Phenomenex). The detector was set at λ 280 nm for GS and 275nm for CR. Data acquisition was performed on class-VP software.

Materials and reagents

Pharmaceutical grades of GS, PR, CF and CR were used and certified to contain 99.7%, 99.7%, 99.7% and 99.9%, respectively.

NQS, boric acid, orthophosphoric acid, potassium dihydrogen phosphate and sodium hydroxide were analytical grade (Sigma-Aldrich, St. Louis, MO). Methanol and acetonitrile were HPLC grade (Sigma-Aldrich, St. Louis, MO).

The pharmaceutical formulations of GS are:

(1) Glucosamine Compound tablets, batch number 606612: each tablet contains 500 mg glucosamine sulphate, 400 mg chondroitin sulphate and 100 mg vitamin C, manufactured by EVA Pharma for Pharmaceuticals and Medical Appliances, S.A.E.
(2) Cartilgin capsules, batch number1229: each capsule contains 250 mg D-glucosamine hydrochloride, 250 mg chondroitin sulphate and 35 mg vitamin C, manufactured by Amoun Pharmaceutical Co. S.A.E El- Obour City, Cairo, Egypt.
(3) Elasticin capsules, batch number 2048005: each capsule contains 250 mg glucosamine sulphate, 200 mg chondroitin sulphate and 30 mg vitamin C, manufactured by Pharo Pharma, Borg Al-Arab Elgededa, A.R.E.
(4) Joflex capsules, batch number 47505: each capsule contains 500 mg glucosamine sulphate, manufactured by Pharaonia Pharmaceuticals for EMAPharm Pharmaceuticals.
(5) Glucosamine capsule batch number 807978: each capsule contains 500 mg glucosamine sulphate, manufactured by EVAPharma for Pharmaceuticals and Medical Appliances, Cairo, Egypt.
(6) Dorofen capsule batch number 1050108: each capsule contains 500 mg D-glucosamine sulphate and 50 mg ginkgo biloba extract, manufactured by South Egypt Co. for Pharmaceuticals and Chemical Industries, Assiut City under license from Liptis Pharmaceuticals.

Somadril tablets (batch number 8EE 1036) were manufactured by Minapharm Pharmaceutical and Chemical industries (Heliopolis, Cairo, Egypt). Each tablet was labeled to contain 200 mg CR, 160 mg PR and 32 mg CF.

The alkaline borate buffer solutions (0.1M and 0.4 M) were made by dissolving boric acid in water and adjusted to the desired pH with 2 M NaOH solution.

Stock solution of NQS was freshly prepared by dissolving 0.2 g in 100 mL distilled water and stored in the dark (a flask coated with aluminum foil) at room temperature.

Chromatographic conditions

The HPLC separation and quantitation were made on a 250 × 4.6 mm (i.d.), 5 μm C_{18} column (Luna, Phenomenex). The mobile phase for GS was prepared by mixing acetonitrile and water in ratio (10: 90, *v*/*v*, pH 7.3 adjusted with 0.1 M NaOH) and UV detection at 280 nm. While mobile phase for CR, PR and CF was prepared by mixing acetonitrile and 20 mM KH_2PO_4 (pH 3.0 adjusted with phosphoric acid) in a ratio 20:80, *v*/*v* and UV detection at 275nm. The flow rate for GS, CR, PR and CF was 1 mL/min. All determinations were performed at ambient temperature. The injection volume was 20 μL. The samples were filtered through 0.45-μm pore size disposable filters. Data acquisition was performed with class-VP software.

Standard solutions

Stock standard solutions of CR, PR and CF were prepared by dissolving 50 mg of each compound in 100 mL methanol; GS was dissolved in 100 mL (80:20 water–methanol).

The standard solutions were prepared by further dilution of the stock standard solution with the specified mobile phase to reach the concentration range of 1–100 μg/mL for GS, 1–150 μg/mL for CR and PR and 1–150 μg/mL for CF.

Derivatization procedures

Different aliquots of the standard solution containing 10–1000 μg of GS and 10–1500 μg of CR were transferred to 10 mL screw cap glass vial, 0.5 mL (100 μg/mL) of NQS and 1 mL borate buffer (pH 8.5, 0.1M) were added. The solutions were allowed to stay for 20 min in a thermostatted oven at 70°C, cooled to room temperature before injection. The content of each vial was transferred to a 10 mL volumetric flask and diluted to volume with mobile phase specific for each compound. Triplicate 20 μL injections were performed for each concentration of GS and CR under the previously described chromatographic conditions.

Sample preparation

GS Formulations

Twenty capsules (tablets in the case of GS compound) were weighed and finely powdered. A portion of the powder equivalent to 50 mg GS was accurately weighed, transferred to a 100-mL volumetric flask and extracted with approximately 80 mL (80:20 water–methanol) in an ultrasonic bath for 30 min. The extract was cooled to room temperature and the solution was diluted to 100 mL with the same solvent and then filtered through a 0.45-μm membrane filter (Millipore, Milford, MA). The first portion of the filtrate was discarded and the remainder was used as stock sample solution.

Different aliquots of sample solutions containing 10–1000 μg GS were transferred to a 10 mL screw cap glass vial and derivatized as described previously and the resulting solutions were chromatographed in triplicate.

Somadril tablets

Twenty tablets were weighed and finely powdered. A portion of the powder equivalent to approximately 50 mg CR, 80 mg PR and 16 mg CF was weighed accurately, transferred to a 100 mL volumetric flask and extracted with approximately 80 mL methanol in an ultrasonic bath for 30 min. The extract was cooled to room temperature and the solution was diluted to 100 mL with the same solvent and then filtered through a 0.45-μm membrane filter (Millipore, Milford, MA). The first portion of the filtrate was discarded and the remainder was used as stock sample solution.

Different aliquots of sample solutions were transferred to a 10 mL screw cap glass vial and derivatized as described previously and the resulting solutions were chromatographed in triplicate.

Results and Discussion

GS is considered as a dietary supplement by the Food and Drug Administration. Knowing its widespread utilization, it would seem essential to control the quality of GS formulations, particularly in dietary supplements. GS structure lacks a UV

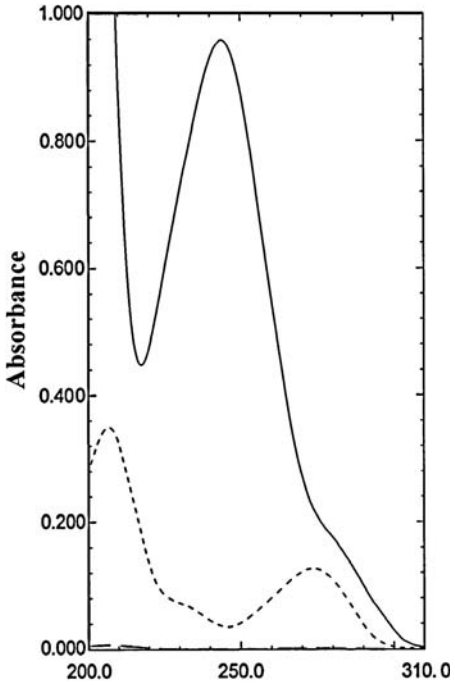

Figure 1. Absorption spectra for 30 μg/mL CR (dashed line), 24 μg/mL PR(straight line), and 4.8 μg/mL CF (straight line).

absorbing chromophore and NQS is a water soluble chromophore that reacts with the primary amino group in an alkaline borate buffer. Similarly, CR has no significant UV absorption, whereas PR and CF have considerable UV absorption (Figure 1). NQS was used with GS and CR as a one-step derivatizing agent producing stable adducts with absorption maximum at longer wavelength, low background noise and higher signal/noise ratio are provided (Figure 2).

Optimization of precolumn derivatization conditions

The process was optimized for high reaction yield and short reaction time. The factors investigated in the screening were the concentration of NQS (A), reaction time (B), reaction temperature (C), pH of borate buffer (D) and ionic strength of the buffer (E).

A quarter-fraction factorial design for five factors at two levels (Table I) was used for screening the effects of the factors on the peak area. For each factor, an upper (+1) and a lower (−1) levels were defined. These levels were based on data from the literature (28) on stoichiometric calculations for the chemical reaction parameters.

To select the most important variables, a quarter-fraction factorial design was used. Only 2^{5-2} (8) experiments have to be performed (Table I).

The effect of each variable on the response is calculated as the difference between the average results at the (+1) level and at the (−1) level of the variable:

$$Ex = \frac{\sum Y(+1)}{n} - \frac{\sum Y(-1)}{n}$$

where Σ Y(+1) and Σ Y(-1) are the sums of the responses where factor x is at its high (+1) and low (−1) level, respectively, and n is the number of times each factor is at the (+1) or (−1) level. Normalized effects ($\%E_x$) can be calculated as $\%E_x = (E_x/Y^{\backslash})^{*}100$ with $Y^{\backslash}$ being the average nominal peak area.

The values of variables' effects indicate that the concentration of NQS, ionic strength and pH of the solution have a negative significant effect on the peak area, while the reaction time and reaction temperature have a positive significant effect.

The optimization was executed with the concentration of NQS at 100 μg/mL, 0.1 M borate buffer, pH at 8.5 for 20 min at 70°C.

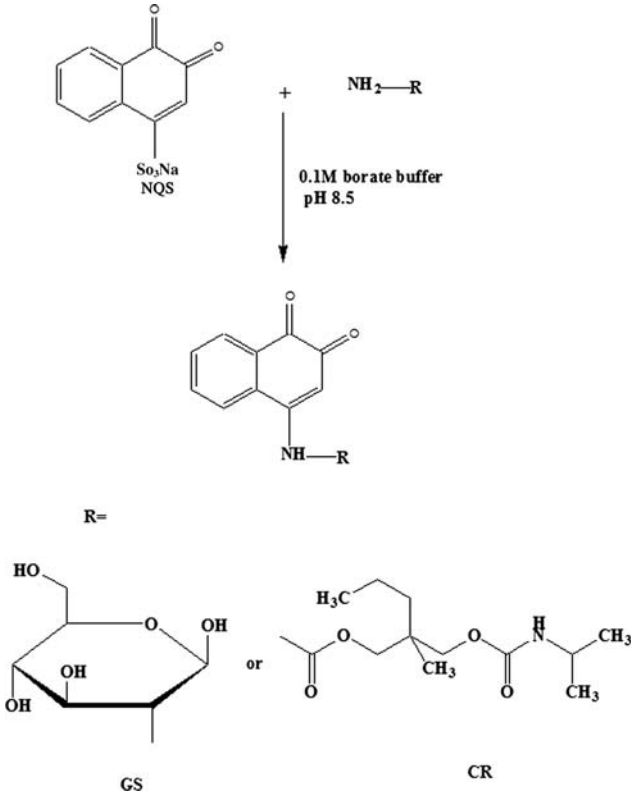

Figure 2. Chemical reaction of NQS with primary amino group containing drugs GS and CR.

Table I
Quarter-Fraction Factorial Design 2^{5-2}_{III}

Experiments	Factors				
	NQS Conc. (mg%) Lower level (−1) is 100 mg% Upper level (+1) is 200 mg%	Reaction time Lower level (−1) is 10 min Upper level (+1) is 20 min	Reaction temperature Lower level (−1) is 50 °C Upper level (+1) is 70 °C	Reagent pH Lower level (−1) is 8.5 Upper level (+1) is 11.5	Ionic strength Lower level (−1) is 0.1 M Upper level (+1) is 0.4 M
	A	B	C	D	E
1	−1	−1	−1	1	1
2	1	−1	−1	−1	−1
3	−1	1	−1	−1	1
4	1	1	−1	1	−1
5	−1	−1	1	1	−1
6	1	−1	1	−1	1
7	−1	1	1	−1	−1
8	1	1	1	1	1

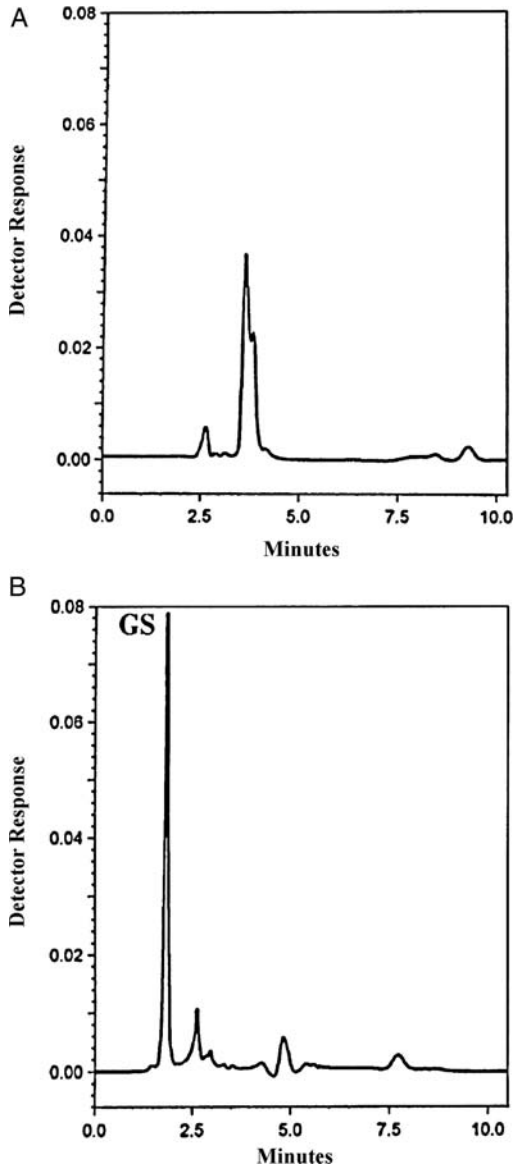

Figure 3. HPLC chromatograms of 20 μL injection of (A) blank reagent; (B) the reaction product of 30 μg/mL GS with 100 μg/mL NSQ.

Optimization of Chromatographic Conditions

GS

Method development was focused on the optimization of sample preparation, chromatographic separation and column detection. GS poses several difficulties during method development. It is a small, polar molecule that has poor retention characteristics in RPLC. Precolumn derivatization of the amino group of GS yields a product that can be detected by UV absorption; during the method development, several chromatographic conditions were assayed to optimize the mobile phase, UV detection and flow rate. Various mobile phases were tested: water– methanol (80:20, *v*/*v*), acetonitrile–water (50:50, *v*/*v*), acetonitrile–20mM phosphate buffer (80:20, *v*/*v*, pH 3.0), acetonitrile–water (20:80, *v*/*v* containing 5mM tetrabutyl ammonium sulphate, pH 7.02).

The test results showed that the solvent system of water could improve the peak shape of the GS naphthoquinone derivative (GS-NQ). The effect of changing the ratio of acetonitrile on the selectivity and retention time of GS-NQ was investigated by using mobile phases containing acetonitrile concentrations in ratio of 5–20%. Ten percent acetonitrile was the best one, giving good resolution between GS-NQ and NQS peaks and the highest number of theoretical plates for GS-NQ peak. Ratios less than 10% resulted in an increase in retention times for excess NQS reagent peaks, whereas ratios higher than 10% resulted in bad resolution between GS-NQ and excess NQS peaks.

The effect of changing the pH of the mobile phase on the selectivity and retention times of GS-NQ was investigated by using mobile phases of pH ranging from 4.0–7.3. A pH value of 7.30 was the most appropriate, giving good resolution between GS-NQ and NQS peaks and the highest number of theoretical plates for GS-NQ peak. pH values lower than 7.30 result in bad resolution between GS-NQ and excess NQS reagent peaks.

Good separation of target compounds and a short run time were obtained using a mobile phase system of acetonitrile and water (pH 7.3 adjusted with 0.1 M NaOH) in the ratio 10:90, v/v and flow-rate 1.0 mL/min and UV detection carried out at 280 nm.

The specificity of the HPLC method is illustrated in Figure 3, where the blank chromatogram (Figure 3A) showed no interfering peaks at the retention time of GS-NQ (Figure 3B). Average retention times ± standard deviation (SD) for GS-NQ were 1.82 ± 0.04 (n = 7 replicates).

CR

The HPLC method was optimized to develop a rapid, simple, accurate, reproducible and sensitive assay for monitoring of the CR-naphthoquinone derivative (CR-NQ), PR and CF. The combined effects of mobile phase composition, pH and potassium dihydrogen phosphate concentration were studied in the determination CR-NQ, PR and CF by HPLC. Preliminary studies were performed by injection of synthetic mixture of the three compounds with NQS reagent.

The mobile phase used for HPLC consisted of two different components, acetonitrile and phosphate buffer. In preliminary experiments, mixtures of acetonitrile and 20 mM phosphate buffer of pH 3.0 were used at a flow rate of 1.0 mL/min. The effect of changing the ratio of acetonitrile on the selectivity and retention times of the test drugs was investigated using mobile phases containing concentrations of 10–40% for acetonitrile. Twenty percent acetonitrile was the best, giving well-resolved peaks and the highest number of theoretical plates. Ratios less than 20% resulted in increase retention times for excess of NQS reagent peaks, whereas ratios higher than 20% resulted in bad resolution between PR, CF and excess of NQS reagent peaks.

The effect of mobile phase pH on the retention times of CR-NQ, PR and CF was studied over the range 2.5–7.0, with 20% acetonitrile in the mobile phase. The chromatographic behavior of CR-NQ was substantially affected by mobile phase pH. The maximum capacity factor (K') was obtained at pH 5–7, with loss of peak symmetry for PR and CF. At pH 2.5, the retention time of CR-NQ was much less, but there was interference between CR-NQ and PR peaks. Between pH 3.0

and 4.0, CR-NQ was better resolved from PR and CF. The most appropriate pH was 3.0, giving well-resolved peaks, the highest number of theoretical plates and reasonable retention time.

The effect of the concentration of potassium dihydrogen phosphate in the mobile phase was studied between 10 and 40 mM. Twenty mM was found to give better resolution of CR-NQ, PR and CF.

On the basis of these investigations, good chromatographic separation of CR-NQ, PR and CF was achieved by use of mobile phase prepared by mixing 20 mM potassium dihydrogen phosphate (pH adjusted to 3.0 by use of phosphoric acid) with acetonitrile in the ratio 80:20 (v/v). The flow rate was 1.0 mL/min. Quantification was achieved by use of UV detection at 275 nm. The specificity of the HPLC method is illustrated in Fig. 4, where the blank chromatogram (Figure 4A) showed no interfering peaks at the retention times of CR-NQ, PR and GF. Figure 4B shows a typical chromatogram for synthetic mixture containing CR-NQ, PR and CF in which the drugs were well separated. Average retention times ($\pm$SD) for CR-NQ, PR and CF were 2.44 $\pm$ 0.01, 4.44 $\pm$ 0.01 and 5.02 $\pm$ 0.03 min ($n = 10$ replicates). Results from system-suitability testing are listed in Table II.

Validation

Linearity

The linearity of the proposed method was evaluated by analyzing seven concentrations of GS and CR, PR, with CF ranging between 1–100 μg/mL for GS, 1–150 μg/mL for CR and PR and 1–150 μg/mL for CF. Each concentration was repeated three times. The assay was performed according to previously established experimental conditions. The calibration graphs were constructed by plotting peak area measured at 280 nm for GS, and 275 nm for CR, PR and CF against corresponding concentrations of each drug.

The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table III).

Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level was evaluated by one-way analysis of variance (ANOVA). An 8 day x 2 replicate design was performed. Statistical comparison of the results was performed using the *P*-value of the *F*-test. Three-univariate ANOVA for each concentration level were made. Because the *P*-value of the *F*-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95 % confidence level.

Range

The calibration range was established through consideration of the practical range necessary, according to each

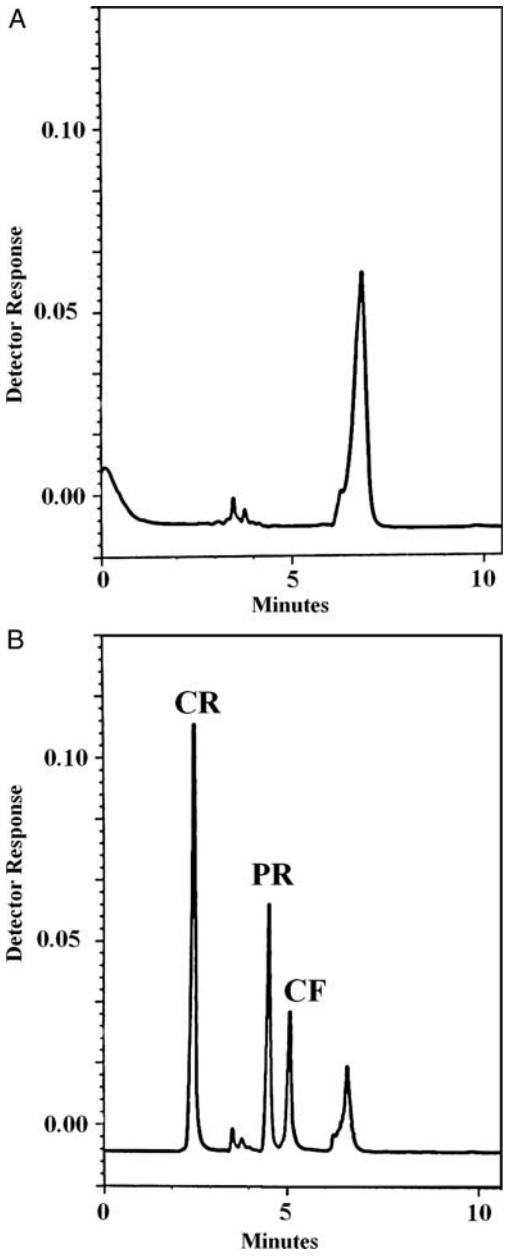

Figure 4. Typical HPLC chromatograms of 20 μL injection of (A) blank reagent; (B) laboratory prepared mixture of reaction product of 31 μg/mL CR with 100 μg/mL NSQ, 25 μg/mL PR and 5 μg/mL CF.

Table II
The System Suitability Test Results of the Developed Method for Determination of CR, PR, and CF. The Retention Time of Unretained Peak is 0.45 min

Comp.	Retention time (min)	Capacity factor ($K^{\backslash}$)	Selectivity α	Resolution R_s	Tailing factor	%RSD of retention time	Plate count
CR	2.44	4.43	2.00 (a_1)	3.07(b_1)	1.04	0.03	56021.52
PR	4.44	8.87	1.14 (a_2)	1.95(b_2)	1.13	0.14	47103.02
CF	5.02	10.15			1.10	0.17	43917.82

a_1, b_1: are α and R_s calculated for CR – PR.
a_2, b_2: are α and R_s calculated for PR – CF.

Table III
Characteristic Parameters of the Calibration Equations for the Proposed HPLC Method for the Determination of GS, CR, PR, and CF

Parameters	GS	CR	PR	CF
Calibration range ($\mu g\ mL^{-1}$)	1-100	1-150	1-150	1-150
Detection limit ($\mu g\ mL^{-1}$)	3.72×10^{-2}	3.59×10^{-2}	3.04×10^{-2}	4.00×10^{-2}
Quantitation limit ($\mu g\ mL^{-1}$)	12.41×10^{-2}	11.97×10^{-2}	10.12×10^{-2}	$13.34x\ 10^{-2}$
Regression equation(Y)[a]: Slope (b)	22.24×10^{3}	25.09×10^{3}	18.19×10^{3}	53.89×10^{3}
Standard deviation of the slope (S_b)	3.53×10^{2}	3.83×10^{2}	2.35×10^{2}	10.07×10^{2}
Relative standard deviation of the slope (%)	1.59	1.53	1.29	1.87
Confidence limit of the slope[b]	$21.91 \times 10^{3} - 22.59 \times 10^{3}$	$24.71 \times 10^{3} - 25.46 \times 10^{3}$	$17.96 \times 10^{3} - 18.42 \times 10^{3}$	$52.91 \times 10^{3} - 54.87 \times 10^{3}$
Intercept (a)	4.61×10^{2}	9.53×10^{3}	-2.80×10^{3}	18.83×10^{3}
Standard deviation of the intercept (S_a)	1.80×10^{2}	3.75×10^{3}	1.49×10^{3}	8.95×10^{3}
Confidence limit of the intercept[b]	$(-16.10 \times 10^{3}) - (17.03 \times 10^{3})$	$(-26.80 \times 10^{3}) - (45.86 \times 10^{3})$	$(-23.41 \times 10^{3}) - (17.82 \times 10^{3})$	$(-69.36 \times 10^{3}) - (10.70 \times 10^{4})$
Correlation coefficient (r)	0.9998	0.9998	0.9998	0.9997
Standard error of estimation	12.25×10^{3}	19.56×10^{3}	11.72×10^{3}	50.33×10^{3}

[a] $Y = a + bC$, where C is the concentration of GS, CR, PR, and CF in $\mu g\ mL^{-1}$ and Y is peak area.
[b] 95% confidence limit.

Table IV
Determination of CR, PR and CF in Laboratory Prepared Mixtures and Pharmaceutical Preparations using the Proposed HPLC Method

Sample	Mean found ± S.D.[a]		
	CR	**PR**	**CF**
Laboratory prepared mixtures	99.53 ± 0.95	99.99 ± 0.70	99.71 ± 1.14
Somadril tablets	100.07 ± 0.93	99.37 ± 1.23	98.90 ± 1.22
Recovery[b]	99.42 ± 1.78	100.08 ± 1.03	99.12 ± 1.09

[a]Mean and S.D., percentage recovery from the label claim amount.
[b]For standard addition of different concentrations of CR, PR and CF.

Table V
Determination of GS in Pharmaceutical Preparations using the Proposed and Published Methods

Sample	Mean found ± S.D.[a]	
	Proposed HPLC method	**Published method [8]**
Glucosamine compound tablets	99.80 ± 0.93	99.21 ± 1.70
t	0.80	(2.18)[b]
F	3.32	(4.28)[b]
Cartilgin capsules	99.64 ± 0.83	98.81 ± 1.05
t	1.64	(2.18)[b]
F	1.60	(4.28)[b]
Elasticin capsules	99.94 ± 0.37	99.69 ± 0.59
t	0.98	(2.18)[b]
F	2.56	(4.28)[b]
Joflex capsules	100.54 ± 0.86	101.10 ± 1.20
t	1.00	(2.18)[b]
F	1.96	(4.28)[b]
Glucosamine capsule	100.09 ± 0.86	100.20 ± 1.60
t	0.17	(2.18)[b]
F	3.48	(4.28)[b]
Dorofen capsules	99.56 ± 1.13	100.93 ± 1.98
t	1.59	(2.18)[b]
F	3.05	(4.28)[b]
Recovery[b] from Glucosamine compound tablets	98.91 ± 1.16	
Recovery[b] from Cartilgin capsules	99.66 ± 1.26	
Recovery[b] from Elasticin capsules	100.43 ± 1.18	
Recovery[b] from Joflex capsules	100.61 ± 1.13	
Recovery[b] from Glucosamine capsules	99.53 ± 1.33	
Recovery[b] from Dorofen capsules	99.20 ± 0.97	

[a]Mean and S.D., percentage recovery from the label claim amount.
[b]For standard addition of different concentrations of GS .

compound concentrations to give accurate, precise and linear results. The calibration ranges of the proposed method are given in Table III.

Detection and quantitation limits. According to ICH recommendations (29), the approach based on the SD of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Table III.

Selectivity. Method selectivity was achieved by preparing seven laboratory-prepared mixtures of CR, PR and CF at various concentrations within the linearity range. The synthetic mixtures were analyzed according to the previous procedures described under the proposed method. Satisfactory results were obtained (Table IV) indicating the high selectivity of the proposed method for simultaneous determination of CR, PR and CF.

Accuracy. The validity of the proposed method for the determination of drug in pharmaceutical formulations was tested by applying the standard addition technique. This study was performed by addition of known amounts of the studied compound to a known concentration of the commercial pharmaceutical products. The results obtained were reproducible with low SDs (0.97–1.78), and the mean recoveries were in the range of 98.91–100.61%. The results reported in Tables 4 and 5 suggested the good accuracy of the proposed method.

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameters. To determine the robustness of the proposed method, experimental conditions such as concentration of NQS and ionic strength of borate buffer were purposely altered. Variation of the NQS concentration by ± 20 μg/mL and ionic strength of borate buffer by 0.05 M did not have a significant effect on the proposed method.

Variation of organic strength of the mobile phase by ± 2% or pH by ± 0.1 has no significant effect on separation. Changing flow rate of the chromatographic method from 0.8 to 1.2 has no significant effect on separation.

Analytical solution stability

The GS solution in methanol–water (20/80, v/v) exhibited no changes for 24 hours when kept at room temperature, or for 15 days when stored refrigerated at 4°C.

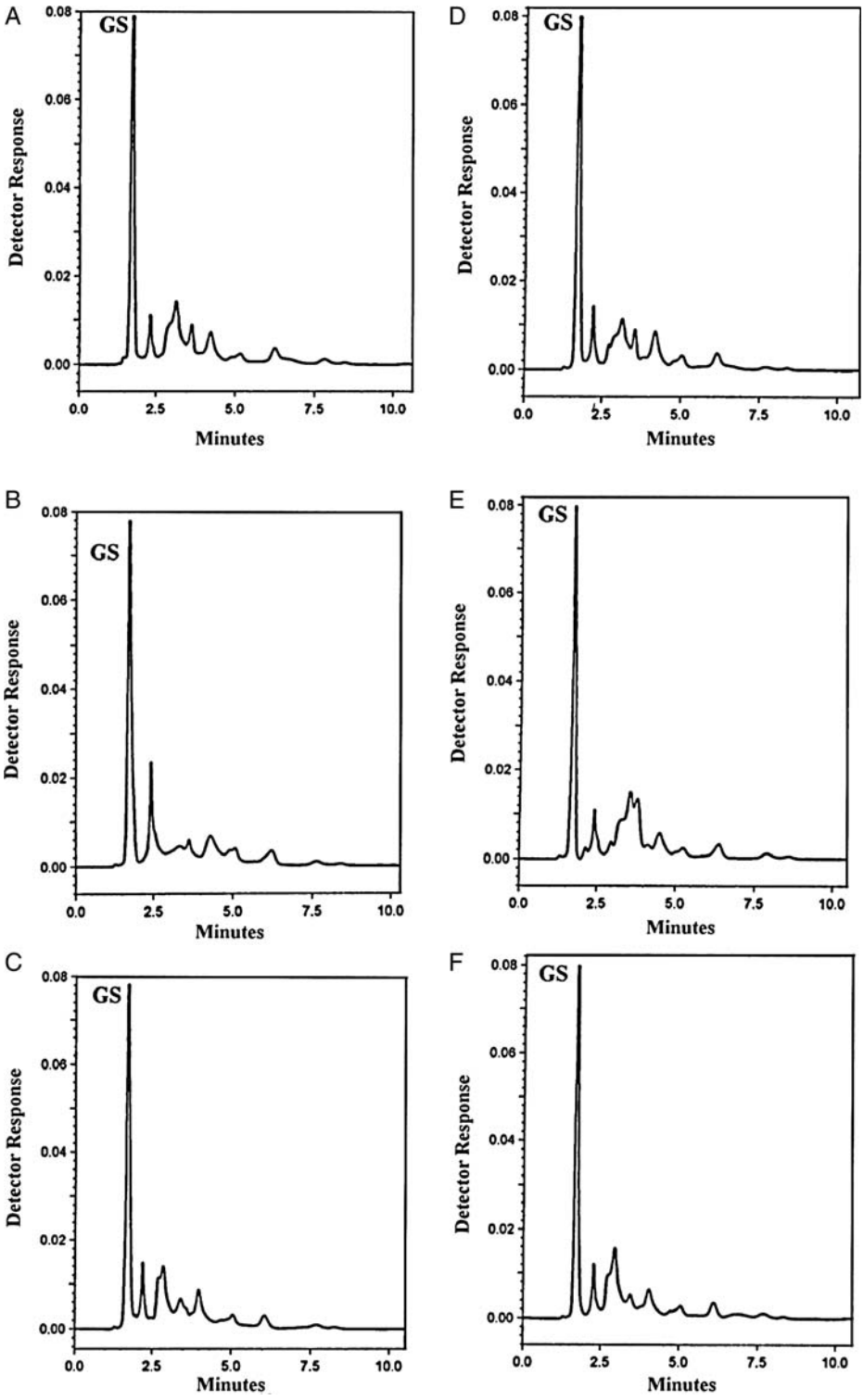

Figure 5. Typical HPLC chromatograms of 20 μL injection of reaction product of 30 μg/mL GS with 100 μg/mL NSQ in (A) Glucosamine compound; (B) Cartilgin; (C) Elasticin; (D) Joflex; (E) Glucosamine; (F) Dorofen pharmaceutical formulations.

CR solution in methanol exhibited no changes for 10 hours when kept at room temperature, or for 3 days when stored refrigerated at 4°C.

The results showed that no significant difference was found among the peak areas at zero time and after 4, 8, 12 and 24 hours at 4°C for GS-NQ and CR-NQ derivatives, which indicated the stability of NQS derivative of GS and CR at 4°C.

PR and CF solution in methanol exhibited no changes for 10 hours when kept at room temperature, and for 5 days when stored refrigerated at 4°C.

Analysis of pharmaceutical formulations

GS formulations

To extract the GS from the dietary supplement and to avoid interference from the other constituents, three solvents were tried: methanol, water and a mixture of methanol–water (20/80, v/v). Water was not chosen alone as a solvent, because although GS is totally soluble in water, so are other substances from formulation excipients or plant extracts, such as sugars.

On the other hand, pure methanol was not chosen because the solubility of GS in this solvent is too weak. The mixture of methanol–water (20/80, v/v) gave the best compromise between good solubilization of GS and its selective extraction.

To investigate the applicability of the proposed method to the determination of GS in pure form or in pharmaceutical formulations, the effect of the presence of some common excipients and coexisting compounds such as starch, talc, lactose and magnesium stearate, chondroitin sulphate, vitamin C, and ginkgo biloba extract that may be present in tablets or capsules was studied. It was found that the common excipients and coexisting compounds did not interfere in the determination.

The developed method was applied for the determination of GS content in six marketed products.

The chromatograms from commercial products showed a good separation of GS-NQ peak from other components present in different pharmaceutical formulations (Figure 5).

Vitamin C could not be assessed by the proposed method because it undergoes extensive hydrolysis in alkaline pH, which is the most suitable pH for derivatizing GS with NQS.

The results were compared to those obtained by the the published HPLC method (8) for GS. Seven replicates determinations were made. Satisfactory results were obtained for GS in good agreement with the label claims in each formulation (Table V). The performance of the proposed method was judged with regards to accuracy and precision by calculating student's *t* -test and *F*-values. At a 95% confidence level, the calculated *t*- and *F*-values do not exceed the theoretical values, indicating no significant difference between the proposed method and the reference method.

CR formulation

The method was used for analysis of CR, PR and CF in tablets. Seven replicate determinations were performed. Satisfactory results were obtained for CR, PR and CF in good agreement with the label claim (Table IV).

Conclusion

In this work, the combination of an optimized precolumn derivatization and RPLC separation for determination of GS and CR in solid dosage formulations was developed and validated. This method was also successfully used for the analysis of GS in various different marketed formulations and used for determination of CR, PR and CF in their ternary mixture while ensuring a quantitative elimination of potential interferences.

An experimental design approach allows the optimal conditions for the determination of these drugs to be found with minimal experiments and trials. The developed method could be of high interest for laboratories of quality control or in the pharmaceutical industry for the routine quantitative analysis of these drugs.

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