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ARTICLES

HPLC Determination of Glucosamine Hydrochloride and Chondroitin Sulfate, Weakly Absorbing in the Near UV Region, in Various Buffer Media

V. M. Kosman, M. N. Karlina, O. N. Pozharitskaya, A. N. Shikov*, and V. G. Makarov

St. Petersburg Institute of Pharmacy, Kuz'molovskii village, Leningrad oblast, 188663 Russia

**e-mail: spbpharm@mail.ru*

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Abstract—Some features of the HPLC determination of two hydrophilic substances that poorly absorb in the UV spectral region (chondroitin sodium sulfate and glucosamine hydrochloride), associated with the absorption in the near UV region (195 nm) of components of buffer solutions with different pH values (1.2, 4.5, and 6.8), are considered. Such solutions are used, for example, in comparative dissolution kinetics tests in pharmaceutical practice. At some pH, the subtraction of the areas of system peaks from the total areas of analyte peaks made it possible to compensate for the negative effect of the solvent. To determine glucosamine hydrochloride in 0.1 M HCl, a procedure was developed and validated, involving the synthesis of *o*-phthalic derivatives. The revealed analytical features are caused by that the solvent of the samples and the eluent do not match; the effect can be most pronounced in measurements in the near UV region and in the determination of ionic compounds.

Keywords: glucosamine hydrochloride, chondroitin sulfate, HPLC, quantitative determination, buffer solutions, comparative dissolution kinetics test

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HPLC is widely used in analytical practice because of its rapidness, sensitivity, selectivity of the corresponding procedures, and the possibility of determination of several analytes simultaneously [1, 2]. At the same time, HPLC is sensitive to various factors and conditions of the analysis, for example, the type of adsorbent and characteristics of analytical columns, the eluent composition, the temperature of chromatographic column, the eluent flow rate, the solvent of sample, etc. [1]. The use of buffer solutions for dissolving the dosed samples significantly affects the results of chromatographic analysis.

The problem of the quantitative determination of substances dissolved in buffer media with different pH arises, in particular, in the comparative dissolution kinetics test (CDKT). This test is aimed at comparing the equivalence of release profiles of active substances in several dissolution media close to the conditions of the gastrointestinal tract [3]. The implementation of CDKT is regulated by a number of Russian [3] and international [4–6] documents and is aimed at reducing the volume of in vivo studies to evaluate the bioequivalence of generic medicinal preparations. The test should be performed in four dissolution media: three buffer media (usually with pH 1.2, 4.5, 6.8) and the medium specified in the regulatory documentation for the reference preparation (prototype) [3, 7]. The execution of the test does not involve a change and/or selection of a solvent, depending on the specific features of the analytical method used.

The guidelines and publications [7–9] describe in detail the test conditions, preparation of dissolution media, time points of sampling, acceptance criteria for dissolution levels and coincidence of release curves (similarity factors), but they contain very limited information on analytical procedures used to determine the concentration of the active substance in the dissolution medium.

When performing CDKT, it is most convenient to use a unified procedure for all dissolution media, which is permissible according to [3] if the technique is applicable in all buffer media. Otherwise, the corresponding procedure should be developed and validated [3]. An additional difficulty arises when such substances should be quantified by HPLC that contain no characteristic chromophores or poorly absorb light in the near UV region.

The goal of the present work was to study the features of the HPLC determination of substances weakly absorbing light in the near UV region on an example of glucosamine hydrochloride and chondroitin sulfate in various buffer media used for CDKT.

EXPERIMENTAL

The test samples in the study were tablet preparations containing glucosamine hydrochloride (500 mg/table) and chondroitin sodium sulfate (500 mg/table) as active components, purchased in the pharmacy network. Glucosamine hydrochloride (USP RS, United States) and chondroitin sodium sulfate (USP RS, United States) were used as reference samples. Solvents and high-purity reagents were used for the preparation of eluents: acetonitrile (grade 0, Kriokhrom, Russia) and methanol (HPLC gradient grade, J.T. Baker, United States); purified water was obtained using a Simplicity Water Purification System (Millipore, United States). We also used sodium 1-octanesulfonate (Khimmed, Russia), phosphoric acid 50% (HPLC grade, Sigma-Aldrich, Germany), triethylamine (Sigma-Aldrich, Germany), *o*-phthalaldehyde (Acros Organics, Germany), 3-mercaptopropionic acid (Aldrich, Germany), and salts and hydrochloric acid (for preparing release media) (Vekton, Russia).

The release rate was evaluated in vitro using a Dissolution test using a DT 600 paddle-type device (Erweka, Germany) at a dissolution medium temperature of 37 ± 1°C, a paddle rotation rate of 75 rpm in the following dissolution media (the total volume of the dissolution medium is 900 mL):

(1) Dissolution medium according to regulatory documentation, distilled water (purified);

(2) Dissolution medium with pH 1.2, 0.1 M HCl;

(3) Dissolution medium with pH 4.5, an acetate buffer solution prepared according to the Russian State Pharmacopoeia XII [10];

(4) Dissolution medium with pH 6.8, a phosphate buffer solution prepared according to the Russian State Pharmacopoeia XII [10].

The concentration of glucosamine hydrochloride and chondroitin sodium sulfate in dissolution media was determined by HPLC. The samples were analyzed using a Shimadzu high pressure chromatograph (Japan) with a μBondapack C18 column (3.9 × 300 mm, 10 μm) (Waters, United States) in the isocratic elution mode. The eluent contained 10 mL of a concentrated buffer solution (10 mL of triethylamine and 13.6 mL of 50% phosphoric acid in 100 mL), 100 mL of acetonitrile, and 1.2 g of sodium octanesulfonate in 1 L; the flow rate of the eluent was 0.6 mL/min; the injected sample volume was 20 μL; and the detection wavelength was 195 nm.

The concentration of glucosamine hydrochloride in samples obtained in a dissolution medium with pH 1.2 was determined by reversed-phase HPLC with UV detection after the synthesis of the products of reaction of glucosamine hydrochloride with *o*-phthalaldehyde [11]. The samples were analyzed using the same high pressure chromatograph with a Luna C_{18} (2) column (4.6 × 150 mm, 5 μm) and a precolumn (3.0 mm) packed with the same adsorbent (Phenomenex, United States) in an isocratic elution mode. The eluent was an acetate buffer solution with pH 5.9 and methanol for liquid chromatography in a volume ratio of 6 : 4; the eluent flow rate was 1.0 mL/min; the injected sample volume was 20 μL; and the detection wavelength was 340 nm.

The chromatograms were recorded and processed using LabSolution software (Shimadzu, Japan). Microsoft Office Excel 2007 software was used for statistical data processing.

RESULTS AND DISCUSSION

Glucosamine is an amino monosaccharide; chondroitin is a high-molecular-weight mucopolysaccharide (Fig. 1). These compounds are not simple samples for the direct HPLC determination because they are both sufficiently hydrophilic and weakly retained on reversed-phase adsorbents and do not have specific chromophores that could provide intense absorption in the UV region. In this case, an eluent containing ion-pair additives (triethylamine and octanesulfonic acid) ensuring the retaining of the analytes should be used for their direct HPLC determination, and the chromatograms should be recorded at 195 nm [12]. Typical chromatograms of the solutions of reference samples of glucosamine hydrochloride and chondroitin sodium sulfate, obtained under the indicated conditions, are shown in Fig. 1.

The CDKT provides an estimate of the kinetics of release of glucosamine hydrochloride and chondroitin sodium sulfate into four different media; therefore, the effect of these solvents on the results of chromatographic determination was tested in a separate series of experiments (Fig. 2). The smoothest baseline was obtained for purified water (Fig. 2a). In the chromatograms for additional dissolution media (Figs. 2b–2d), significant system peaks are recorded, primarily due to the absorption of various anions and cations in the near UV region (195 nm) used for detection. For the acetate buffer solution with pH 4.5 in the elution region of the target components (2.5–3.5 min), insignificant system peaks were observed (Fig. 2c), the areas of which were subtracted from the peak areas of the analytes. For the phosphate buffer solution with pH 6.8, the subtraction of the area of the coeluted background system peak was used only for glucosamine hydrochloride (Fig. 2d). For 0.1 M HCl and the phosphate buffer solution with pH 6.8, the effect of the solvent on the peak area of chondroitin sodium sulfate is negligible. At the same time, in 0.1 M HCl, the peak of glucosamine hydrochloride is overlapped by the peak of the solvent, probably, the chloride ion (Fig. 2b). Therefore, in order to determine glucosamine hydrochloride in the samples obtained for the dissolution medium with pH 1.2, another procedure should be proposed.

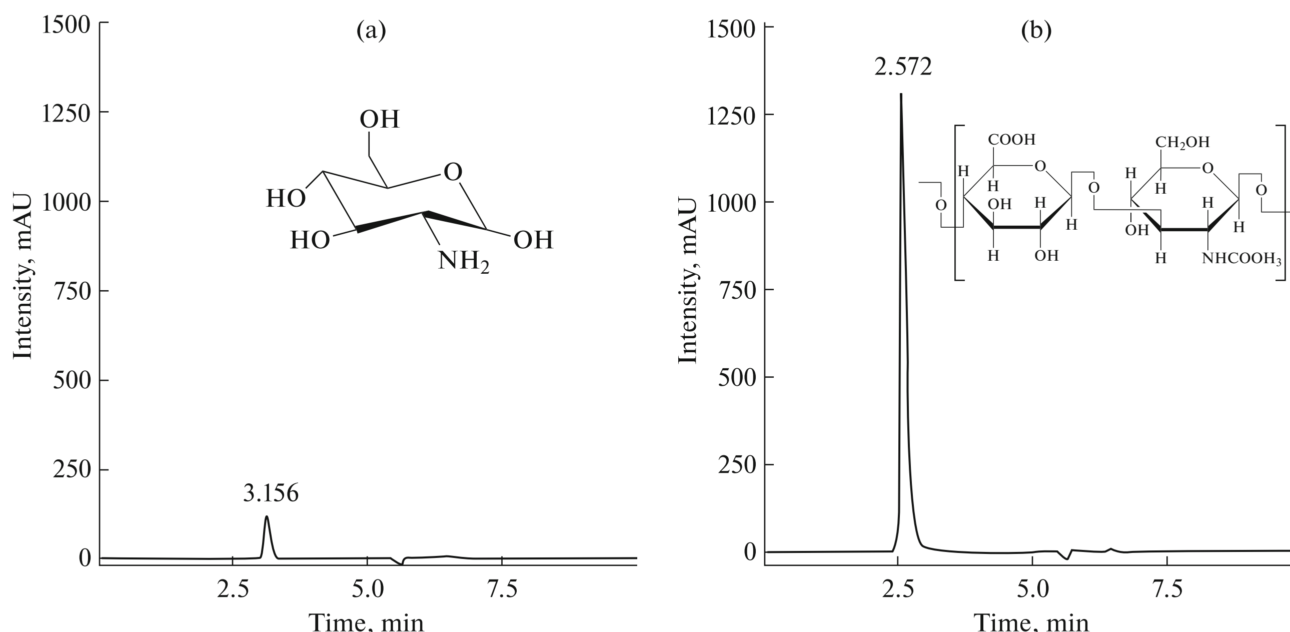

Fig. 1. Typical chromatograms of the reference samples of (a) glucosamine hydrochloride (2-amino-2-deoxy-beta-D-glucopyranose as a hydrochloride) and (b) chondroitin sodium sulfate (chondroitin-4-(hydrogen sulfate) as a sodium salt); λ = 195 nm.

The concentration of glucosamine hydrochloride in samples obtained in the dissolution medium with pH 1.2 was determined by reversed-phase HPLC with UV detection after obtaining the products of the reaction of glucosamine hydrochloride with *o*-phthalaldehyde [11]. The products of the derivatization reaction have a specific UV spectrum, and they are capable of fluorescence, which enables the use of appropriate detectors. In addition, the maximum absorption of the reaction products is in the region of ~340 nm, where the interference of nontarget components of the samples (primarily, their solvents—buffer solutions) is much lower.

A dominant peak of the β-anomer of glucosamine hydrochloride (used for further quantitative analysis) and the peak of its α-anomer are recorded in the chromatogram of the reference sample of glucosamine hydrochloride (Fig. 3). The peaks of the target analyte, as well as any additional interfering components, were not recorded in the chromatogram of the reagent mixture for derivatization. This indicates the specificity of the procedure and its applicability for the analysis of samples obtained for a dissolution medium with a pH of 1.2, in contrast to the direct HPLC determination discussed above.

The procedures for determining glucosamine hydrochloride and chondroitin sodium sulfate by direct HPLC method and by the procedure with the synthesis *o*-phthalic derivatives (for glucosamine hydrochloride) were validated in accordance with recommendations [13–15] for parameters specificity (Figs. 1–3), linearity, accuracy, and precision (Table 1). Satisfactory results were obtained for all the listed parameters.

The approach of subtracting system “interfering” peaks related to the solvent and/or placebo is described for the HPLC procedures for standardization of medicinal preparations [8]. Its application is important both for the qualitative interpretation of chromatograms (for example, which peaks to disregard, as they relate to impurities) and for quantification in the case of overlapping and/or poorly separated peaks. The subtraction of system peaks in quantitative analysis is analogous to the standard addition method, based on the “analytical signal-to-addition weight” ratio, which involves subtracting the peak area of the analyte in the initial sample from the peak area of the analyte in the same sample after addition of the known (standard) additive.

This technique can be implemented by subtracting the placebo/solvent chromatogram from the chromatogram of the test sample using appropriate software functions or by subtracting the system peak area from the peak area of the analyte, determined in advance by several repeated runs. The first option is based on the selection of one specific chromatogram of a blank sample for subtraction. In the latter case, it is possible to average the subtracted area of the system peak by the results of several runs, which ensures a greater reproducibility of the result. Regarding the example under discussion, the second method was used: when processing chromatograms, additional constants corresponding to the areas of the system peaks were introduced into the calculation formulas.

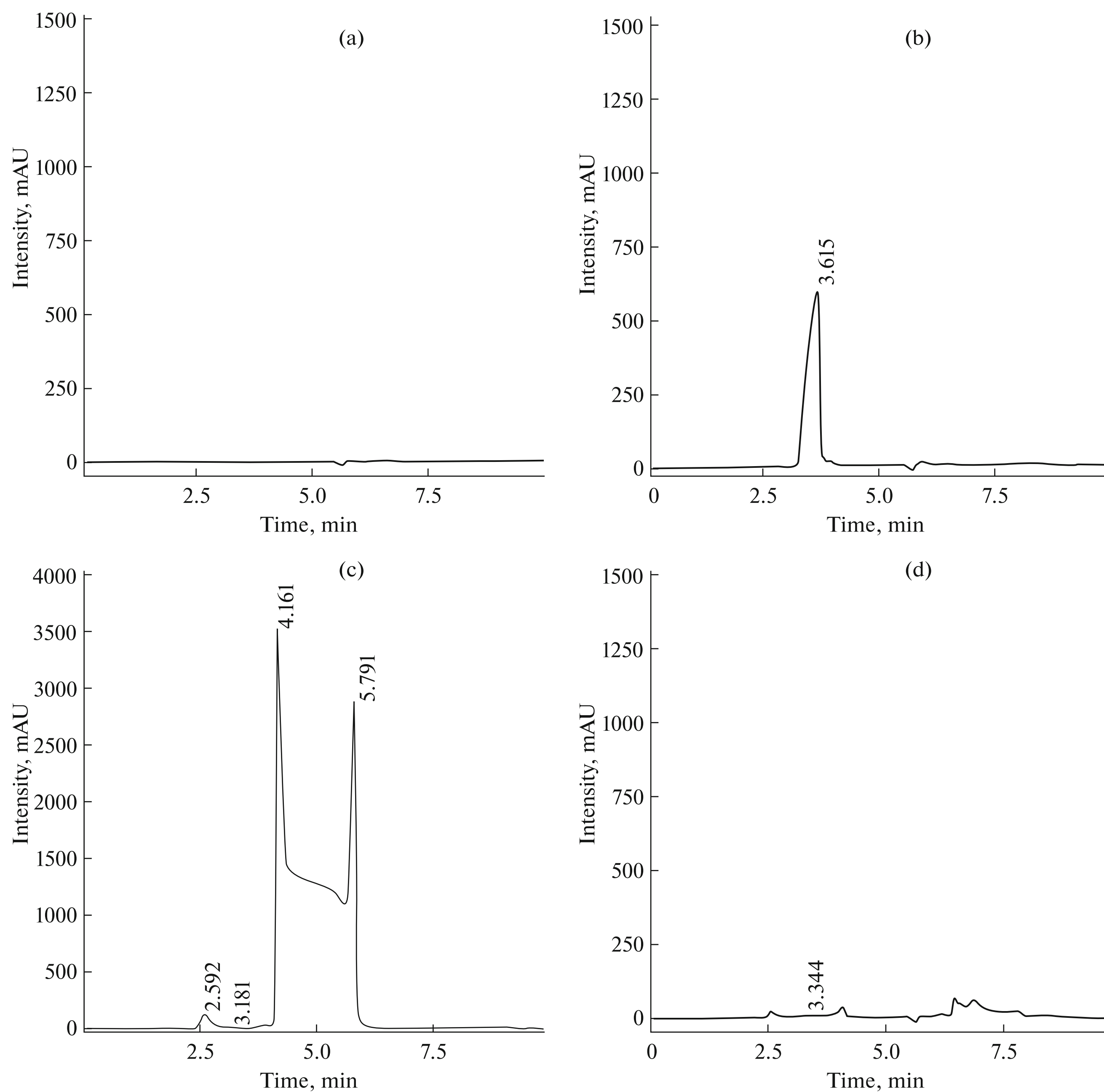

Fig. 2. Typical chromatograms of dissolution media: (a) water, (b) 0.1 M HCl, (c) acetate buffer solution with pH 4.5, and (d) phosphate buffer solution with pH 6.8; $\lambda = 195$ nm.

To confirm the correctness of the application of this method, the accuracy of determination was examined by the standard addition method for model solutions of analytes in appropriate buffer media (Table 2). The systematic error in all cases did not exceed 0.7%, which indicates the adequacy of the approach applied.

The aspects related to the cause of the occurrence of system peaks in an HPLC system and the selection of a solvent for the dosed samples deserve separate discussion. According to Sadek [16, 17], the appearance of system peaks is most probable when working in those spectral regions where the optical density of the eluent is significant (primarily, in the near UV region). The presence of system peaks, their magnitude, and retention time depend on the complex relationship between the sample matrix, the injected sample volume, the detection wavelength, the composition of the mobile phase, and the type of adsorbent [16]. The best way to minimize the number and height of system peaks is to dissolve, as possible, the sample in the eluent and to use such settings of the detector (sensitivity level, wavelength), at which the solvent of the sample

and the mobile phase do not give at all or yield a very low signal [17].

Sadek considers that one of the reasons for the difficulties in the HPLC analysis is the mismatch in the composition of the solvent of sample and the eluent [16, 17], which manifests itself in the form of changes in the shape of peaks, retention times, and quantitative results. The use of the eluent to dissolve the sample is generally accepted in HPLC [1, 18–20]. If this is not possible, it is recommended that one of the eluent components or a compatible solvent be selected for dissolution [18]. When determining the active substances in various dosage forms, it is important that the solvent ensure the most complete extraction of the detectable component from the matrix with minimal dissolution of auxiliary substances [1]. However, these recommendations do not apply to the CDKT, since the solvent of the sample is determined by the buffer solution used as a dissolution medium. In the above example of the determination of glucosamine hydrochloride and chondroitin sulfate, the absorption of the components of buffer solutions at the selected detection wavelength is significant, and the wavelength change is irrational due to the properties of the compounds to be determined.

Additional complications may occur for ionic analytes. First, the UV spectra of many compounds depend on the solvent and pH of the medium, which is related to ionization (the formation of protonated/deprotonated forms) and other chemical transformations (association, complexation, decomposition, oxidation, etc.) of the analytes [1, 21]. In addition, the protonated and deprotonated forms of such compounds are differently retained in HPLC, and when working in the pH range close to pK_a, several forms can exist simultaneously in solutions, which is manifested in the chromatogram in the form of diffuse, asymmetric peaks or the appearance of several peaks of the analyte [1]. A change in the pH of the eluent when injecting samples with a very different pH value can lead to similar changes in the chromatogram, making the selected analysis conditions unacceptable and necessitating a significant or complete reworking of the procedure for each dissolution medium. This should be taken into account when planning works on the implementation of the CDKT. An increase in the volume of experimental work is due not only to the increase in the number of samples [7], but also to the potential need to develop and validate several analytical procedures (in the most unfavorable case, for each dissolution medium). We emphasize that this task may not have an acceptable solution (in terms of time and labor), and the procedure for excluding any dissolution medium from the CDKT is not foreseen in [3] (only nonequivalence of the profiles associated with low solubility or instability in one of the media is allowed). A similar example is described in [22], where the active substance proved to be unstable in one of the dissolution media (at pH 6.8), and the dissolution medium occurred to be unsuitable for the CDKT of this compound.

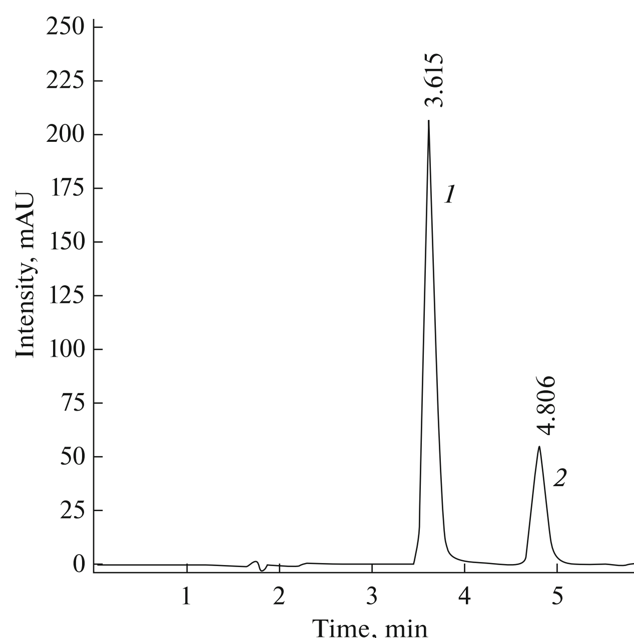

Fig. 3. Typical chromatogram of the reference sample of glucosamine hydrochloride after derivatization: (*1*) β-anomer and (*2*) α-anomer of glucosamine hydrochloride; λ = 340 nm.

CONCLUSIONS

Examples of glucosamine hydrochloride and chondroitin sulfate show the features of the quantitative determination by HPLC in various buffer media of hydrophilic substances weakly absorbing in the UV spectral region. Significant system peaks were recorded in chromatograms of the dissolution buffer media (0.1 M HCl, acetate buffer solution with pH 4.5 and phosphate buffer solution with pH 6.8), related primarily to the absorption of various anions and cations in the near UV region (195 nm) used for detection. In some cases (for example, both analytes with the use of an acetate buffer solution with pH 4.5 or glucosamine hydrochloride with the use of a phosphate buffer solution with pH 6.8), the subtraction of the areas of system peaks from the total area of the analyte peak was applied, enabling the compensation for the negative (overestimating) effect of the solvent. Since in 0.1 M HCl, the peak of glucosamine hydrochloride is overlapped by a solvent peak, a procedure was developed and validated to obtain its *o*-phthalic derivatives. The revealed features are due to the mismatch of the solvent of samples and the eluent; they can be most pronounced upon detection in the near UV region and in the determination of ionic compounds in the pH range close to their pK_a. These factors must be taken into account in the quantitative HPLC determination

Table 1. Validation parameters of procedures for determining glucosamine hydrochloride and chondroitin sodium sulfate in the release medium

Parameter	Value for glucosamine hydrochloride	Value for chondroitin sulfate	Value for glucosamine hydrochloride (using *o*-phthalic derivatives)
Range of linearity, μg/mL	10–1000	5–630	5.6–672
Regression equation*	$Y = 723X - 889$	$Y = 17691X + 8832$	$Y = 3601X - 6320$
Correlation coefficient r, no smaller than 0.9984	0.9998	0.9985	0.9996
Accuracy (%) for three concentration levels (1–3**)			
Level 1	0.2	0.3	0.4
Level 2	0.1	−0.5	0.5
Level 3	−0.7	−0.8	−1.0
Mean ($\|\delta\|$, %), no more than 0.95	0.3	0.5	0.6
Precision (%) not more than the maximum permissible uncertainty of the result (Δ_{As}, 2.96%) for three concentration levels (1–3**)			
Level 1	0.2	1.1	0.2
Level 2	0.1	0.2	1.0
Level 3	1.0	0.2	2.7

* Y is the area of the analyte peak; X is the analyte concentration, μg/mL.
** Concentrations (μg/mL) of solutions of glucosamine hydrochloride for Levels 1–3 are 620, 450, and 350; for solutions of chondroitin sulfate, they are 630, 560, and 400; and for solutions of glucosamine hydrochloride (using *o*-phthalic derivatives), they are 672, 560, and 280, respectively.

Table 2. Evaluation of the accuracy of the determination of glucosamine hydrochloride and chondroitin sulfate using the subtraction of system peaks

Added, μg/mL	Found, μg/mL (average value, $n = 3$)	Error	
		μg/mL	rel. %
Glucosamine hydrochloride, pH 4.5			
620	622.5	+2.5	0.40
450	447.8	−2.2	−0.49
350	351.9	+1.9	0.54
		Mean ($\|\delta\|$, %)	0.48
Chondroitin sulfate, pH 4.5			
630	633.8	+3.8	+0.60
500	497.5	−2.5	−0.50
400	403.4	+3.4	0.85
		Mean ($\|\delta\|$, %)	0.65
Glucosamine hydrochloride, pH 6.8			
620	617.7	−2.3	−0.37
450	448.3	−1.7	−0.38
350	353.3	+3.3	0.94
		Mean ($\|\delta\|$, %)	0.56

with the detection of analytes in various buffer solutions in the UV region of the spectrum. They can also limit the application of the CDKT.

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