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A novel derivatization-free method of formaldehyde and propylene glycol determination in hydrogels by liquid chromatography with refractometric detection

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ABSTRACT

The paper describes the development and validation of a new derivatization-free liquid chromatography method for simultaneous determination of propylene glycol and formaldehyde in the formulations containing formaldehyde-releasing preservative. Highly swollen hydrogel made of poly(ethylene glycol)-protein conjugates was taken as a model formulation for integration of the propylene glycol and the diazolydinyl urea as formaldehyde releaser. The method is shown to be simple and selective and, more importantly, allows determining an existing level of formaldehyde at the moment of analysis instead of all available formaldehyde that might be released during chemical derivatization. After liquid extraction the propylene glycol (PG) and formaldehyde (FA) amounts are determined chromatographically on a Shodex SH 1011 ligand-exchange column using 0.01 M sulfuric acid mobile phase, a flow rate of 1.0 ml/min and RI detection. The assay is validated showing good linearity, precision, and accuracy. The limits of detection of formaldehyde and propylene glycol in the analyzed solutions were estimated to be 25 ng and 87 ng, respectively. This analytical assay is considered useful for product stability studies and in developing new formaldehyde releaser-containing formulations where the concentration of formaldehyde is a presumable subject of labeling requirements. This method can also provide a rapid and convenient alternative to gas chromatography method of propylene glycol quantification.

1. Introduction

Hydrogels represent versatile galenic form for topical drug administration and wound dressing application. Previous reports on the poly(ethylene glycol)-protein hybrid hydrogels used in this study have characterized the material in terms of microstructure [1], drug delivery potential [2] and evaluated the hydrogel-based product as a moist wound dressing [3]. Successful and user-safe exploitation of the hydrogel-based devices in both cosmetic and wound management application requires the highly swollen matrix to possess antimicrobial protection, which is achieved by adding a preservative to the formulation.

In this work the poly(ethylene glycol)-protein hydrogel was integrated with a diazolydinyl urea-containing preservative in attempt to verify compatibility of the hydrogel and the preservative and carry out preliminary evaluation of the dose-dependent antimicrobial effectiveness of the formulation. The preservative chosen is marketed under the trademark of Liquid Germall® Plus (LGP) and comprises diazolydinyl urea (DU), iodopropynyl butylcarbamate (IPBC), and propylene glycol (PG). The composition of LGP is reported to exhibit a synergistic preservative effect against broad spectrum of Gram-positive and Gram-negative bacteria, yeasts, and mold [4]. According to the manufacturer, LGP adequately preserves a variety of formulations at the use levels of 0.1–0.5% [5]. Diazolydinyl urea included in the LGP formulation is unstable in a solution and degrades to form free formaldehyde (FA). This property of DU necessitates a strict control over the composition of a DU-containing formulation, since according to the regulations, if the product contains more than 0.05% FA, the label should state "contains formaldehyde" [6]. For example, when LGP is used at the maximum recommended concentration of 0.5%, the actual composition of a formulation comprises 0.3% PG, 0.198% DU, and 0.002% IPBC. The maximal theoretical concentration of FA that can be released from 0.198% DU in such formulation may reach 0.064% which is above non-declared level. On the other hand, low content of the preservative may not be effective against microbial contamination. Therefore, to guarantee both effective and safe use of DU-containing preservatives, the development of sensitive and reliable method for the analysis and quantification of DU and FA in pharmaceutical and cosmetic products is of great importance.

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FA is typically assayed using dinitrophenyl hydrazine, methylbenzothiazolone hydrazone, acetylacetone or some other toxic reagents derivatization followed by GC, HPLC or colorimetric analysis [7–15]. These methods often require harsh conditions which can provoke degradation of residual non-hydrolyzed DU, thus artificially increasing the level of free FA.

Besides FA, propylene glycol (PG) is another component which often requires quantification in pharmaceutical products. The PG assay in pharmaceutical formulations is typically performed by GC, reversed phase chromatography with precolumn derivatization or by residual titration after oxidation by periodic acid [16,17]. An HPLC method for quantification of free PG using ion-exchange column and pulsed amperometric detection was described [18]. PG concentration in tablets can also be measured by indirect spectrophotometric method after reaction with dye marker, but this method was not adapted to other types of formulation [19]. To the best of our knowledge, no method is reported suitable for simultaneous PG and FA analysis.

The present paper reports experimental data on the preservative effectiveness of the LGP-containing hydrogel and describes the development and validation of a new, simple liquid chromatography method of simultaneous FA and PG determination with the RI detection.

2. Materials and methods

2.1. Chemicals

Poly(ethylene glycol)-protein hydrogels for the study were produced by BioArtificial Gel Technologies Inc. (Montreal, Canada). The details of the hydrogel synthesis have been published elsewhere [20].

Diazolydinyl urea, 95% (DU), 3-iodo-2-propynyl *N*-butylcarbamate, 97% (IPBC), allantoin, sodium chloride, sodium hydrogen phosphate (Na_2HPO_4), phosphoric acid, 85% (w/w), disodium EDTA were obtained from Sigma–Aldrich (MO, USA). Reagent grade formaldehyde 37% (w/w) was obtained from J.T. Baker (NJ, USA). Methanol and sulfuric acid 96.5% (w/w) were of HPLC quality and were supplied by J.T. Baker (NJ, USA). Liquid Germall® Plus containing 60% (w/w) propylene glycol, 39.6% (w/w) diazolidinyl urea, and 0.4% (w/w) 3-iodo-2-propynyl *N*-butylcarbamate was obtained from Sutton Laboratories (NJ, USA). Pharmacopoeial quality propylene glycol was obtained from Riedel-de Haen (Seelze, Germany).

2.2. HPLC analysis

Analysis was carried out on a Waters HPLC system (Milford, MA, USA) equipped with a Waters 600E pump, Waters 410 Refractometric Detector, Waters 996 PDA detector, and Waters 717 Autosampler. Data acquisition was performed using a Millenium v3.5 chromatography software package. The chromatograms were smoothed by 31-point Savitzky–Galey algorithm.

Chromatographic separation was performed using a ligand-exchange column (Shodex SH-1011, 300 mm × 8.0 mm i.d., 7 μm particle size) at a temperature of 60 °C. The mobile phase consisted of 0.01 M H_2SO_4 solution, degassed by vacuum filtration through a 0.22 μm nylon membrane filter. The flow rate was 1.0 ml min^{-1}. The injection volume was 100 μl with 16 min of analysis.

2.3. Preparation of stock hydrogel preparations

Hydrogel preparations containing preservatives were prepared using standard compounding protocols of BioArtificial Gel Technologies Inc. The concentrations of LGP were varied in order to achieve desired preservative effectiveness of the hydrogel and investigate the performance of the analytical method for the FA and PG assays.

Briefly, the solutions containing 0.10%, 0.25%, 0.50%, and 0.75% LGP (w/v) were prepared by dissolution of the weighed quantity of LGP in the buffer solution containing EDTA at pH 5.5. The formulated hydrogels were obtained by incubation during 48 h in the LGP solution. The LGP solution was frequently changed to completely equilibrate the composition of the hydrogels. The formulated hydrogels were sealed in the plastic pouches and stored at ambient temperature.

Several experimental lots of the hydrogel formulations containing 0.5% LGP (w/v) were produced in the manufacturing department and also analyzed by the proposed method.

2.4. Standard solutions for HPLC analysis

Exact concentration of formaldehyde was determined using an assay for the formaldehyde 37% solution as described in the British Pharmacopoeia (2007) [21]. Formaldehyde was reacted with iodine solution and the excess of iodine was back-titrated with sodium thiosulfate using starch as an indicator. The measured concentration of formaldehyde was 39.3% (w/w).

The formaldehyde stock solution (0.35%, w/v) was prepared by diluting 8.40 ml of 39.3% formaldehyde solution in a 1000-ml volumetric flask with mobile phase. Standards were prepared by spiking 0.01 M H_2SO_4 with the stock solution of formaldehyde and consecutive dilutions to the final formaldehyde concentrations of 285, 140, 70, 35, 14, and 1 μg/ml. Since formaldehyde is relatively unstable substance, all stock and standard solutions as well as samples were stored in the dark place at +5 °C and used within 10 h after preparation.

The propylene glycol stock solution (2.50%, w/v) was prepared by dissolving 2.50 g of propylene glycol in 100 ml volumetric flask in 0.01 M H_2SO_4. Standards were prepared by spiking 0.01 M H_2SO_4 with the stock solution of propylene glycol and consecutive dilutions to the final propylene glycol concentrations of 1000, 500, 250, 125, 50, and 5 μg/ml.

2.5. FA and PG assay of the hydrogel preparations

Hydrogel slabs were dabbed with napkin paper to remove excess of liquid from the surface. The hydrogel was cut into small pieces to facilitate extraction. One gram of the crashed gel was placed in a plastic vial and 4.5 ml of 0.01 M sulfuric acid was added. Tightly sealed tubes with the gel samples were vortexed for 20 s each 60 min during 5 h. High water content of the hydrogel (90–96% (w/v) of water) allowed achieving fast and homogeneous redistribution of analytes in complete volume. An aliquot of the supernatant was filtered through 0.22 μm nylon filter and subjected to LC analysis. Each solution prepared was injected by triplicate. The extract solutions were stable at 5 °C for at least 10 h.

Concentration of FA and PG in hydrogel (C_{gel}, w/v) was calculated by formula (1):

$$C_{gel} = C_{an}\frac{4.5 + m_g\rho^{-1}}{m_g\rho^{-1}}, \quad (1)$$

where C_{an} is the concentration of FA (PG) in analyzing solution, % (w/v); m_g is the weight of the gel sample taken for analysis, g; ρ is the gel density (1.013 ± 0.005 g cm^{-3}) [22].

2.6. Preservative effectiveness of the hydrogel preparations

The preservative effectiveness of the hydrogel was tested using the cultures of *Candida albicans* (ATCC 10231), *Aspergillus niger*

(ATCC 16404), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), and *Pseudomonas aeruginosa* (ATCC 9027), which represents bacteria, yeast, and molds according to the USP recommendation [23].

In the tests, 15 g of crashed hydrogel were inoculated with standardized inoculum of the aforementioned microorganisms. Microorganisms were incubated and sampled at varied time intervals. The number of colony forming units (CFU) was determined by plate-count procedure for each microorganism in the control (standardized inoculum), LGP-free hydrogel and hydrogel containing 0.1% and 0.5% of LGP. The efficacy of the formulation was estimated by determining the "log reduction" parameter, i.e. the difference between initial and actual bacteria counts in the sample. The log reduction of not less than 2 from the initial count at 14 days and no increase from the initial calculated count at 14 and 28 days for yeast and molds were set as criteria of the antimicrobial effectiveness of the formulation [23].

2.7. HPLC method validation

2.7.1. Specificity

The specificity was evaluated by analyzing blank LGP-free hydrogels. Additional experiments were carried out to check the absence of interference from hydrogel components and potential degradation products of DU (allantoin, 4-hydroxymethyl-2,5-dioxoimidazolidin-4-yl)-urea).

(4-Hydroxymethyl-2,5-dioxoimidazolidin-4-yl)-urea (HU), the main degradation product of DU, was prepared by incubation of DU in 0.2 M ammonium hydroxide for 3 h [24].

2.7.2. Linearity

Six concentration levels of FA (285–1 μg/ml) and PG (1000–5 μg/ml) were used for system calibration. The linearity was evaluated by the least square regression method with duplicate determinations at each concentration level in three different days.

2.7.3. Precision

The precision of the method was characterized by intra-day and inter-day precision. The injection reproducibility was evaluated by six replicate injections of the FA standard solution (20 μg/ml) and an extract of the hydrogel formulation containing 0.1% LGP. The method repeatability was studied by repeating the assay 9 times in the same day (intra-day precision). The intermediate precision was evaluated by analyzing two samples sets with nine samples in each set on different days (inter-day precision). The intra-day and inter-day variation for determination of FA and PG was carried out at three different levels corresponding to 0.25%, 0.50%, and 0.75% of LGP (w/v) in the hydrogel formulation.

2.7.4. Accuracy

The accuracy was determined by the recovery test of the analytes in the LGP-laden hydrogels with spikes of FA and PG (method of standard additions). Known amounts (about 50% of the representative level) of FA and PG were added to 1-g hydrogel samples formulated with 0.25%, 0.50%, and 0.75% LGP (w/v). All samples were prepared in triplicate and analyzed by LC.

2.7.5. Limit of detection and limit of quantification

Limits of detection (LOD) and limit of quantification (LOQ) of FA and PG were calculated based on a standard deviation of the response near quantification limit and the slope of the calibration curve according to Eqs. (2) and (3):

$$\mathrm{LOD} = 3\frac{\mathrm{SD}}{S}; \tag{2}$$

$$\mathrm{LOQ} = 10\frac{\mathrm{SD}}{S}; \tag{3}$$

where SD is the standard deviation of the *y*-intercept and *S* is the slope of the linearity curve in the low concentration range of an analyte.

Table 1
Antimicrobial effectiveness ("log reduction" values) of the hydrogels integrated with different concentrations of LGP.

Formulation	Days				
	1	2	7	14	21
Candida albicans					
Control	−0.72	−0.34	−0.58	−0.30	−0.30
LGP 0%	−0.18	−0.24	0.59	4.70	4.70
LGP 0.1%	−0.20	0.95	4.70	4.70	4.70
LGP 0.5%	4.70	4.70	4.70	4.70	4.70
Pseudomonas aerugenosa					
Control	−1.38	−1.17	−1.04	0.94	0.94
LGP 0%	2.58	2.55	5.04	5.94	5.94
LGP 0.1%	3.27	5.94	5.94	5.94	5.94
LGP 0.5%	5.94	5.94	5.94	5.94	5.94
Staphylococcus aureus					
Control	−0.27	−0.49	0.48	1.81	1.81
LGP 0%	0.85	0.76	2.27	3.90	5.37
LGP 0.1%	0.17	6.81	6.81	6.81	6.81
LGP 0.5%	3.02	6.81	6.81	6.81	6.81
Escherichia coli					
Control	−0.66	−0.77	−0.53	1.64	1.64
LGP 0%	0.53	0.20	1.14	1.94	3.74
LGP 0.1%	1.32	2.12	5.87	6.64	6.64
LGP 0.5%	2.44	6.64	6.64	6.64	6.64
Aspergillus niger					
Control	0.52	1.66	0.30	0.23	−0.92
LGP 0%	0.14	0.15	0.18	0.52	0.49
LGP 0.1%	0.26	0.76	4.08	3.60	4.08
LGP 0.5%	4.08	4.08	4.08	4.08	4.08

3. Results and discussion

3.1. Preservative effectiveness of the LGP-containing hydrogels

As shown in Table 1, all tested formulations of hydrogel meet current USP criteria after 14 days of incubation. Notably, no *C. albicans* or *P. aeruginosa* was recovered at 14 days even in the LGP-free hydrogel, indicating that the formulation of the hydrogel itself exerts some antimicrobial activity. This result is not unexpected, given a well-known potential of EDTA included in the hydrogel formulation in provoking loss of viability of the tested microorganisms

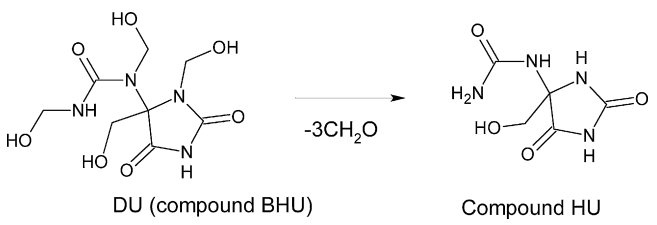

Fig. 1. Schematic illustration of DU hydrolysis in water. DU consists a 1-(3,4-bis-hydroxymethyl-2,5-dioxo-imidazolidin-4-yl)-1,3-bis-hydroxymethyl-urea (BHU) as a principle compound [24]. Complete degradation of BHU generates three molecules of FA and (4-hydroxymethyl-2,5-dioxoimidazolidin-4-yl)-urea (HU).

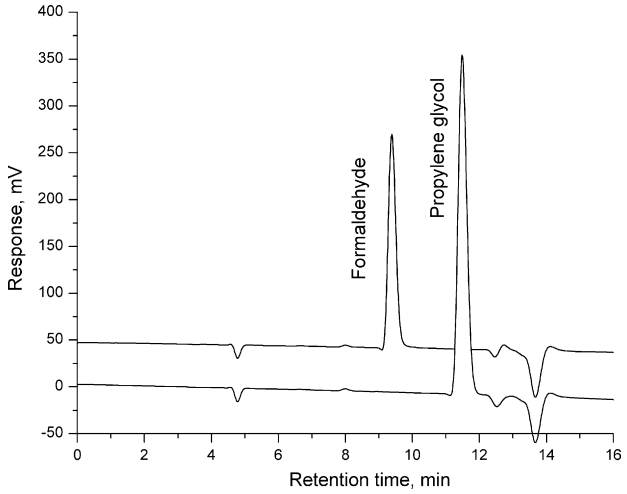

Fig. 2. Overlaid chromatograms of FA and PG standard solutions prepared in 0.01 M H_2SO_4. Chromatography conditions are detailed in Section 2.

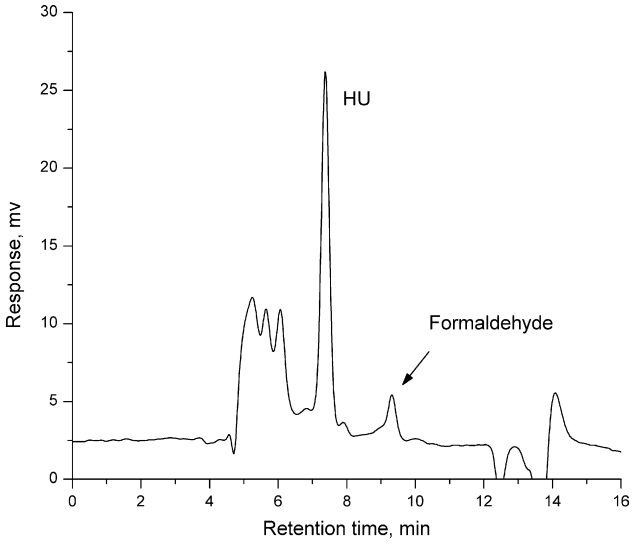

Fig. 4. Chromatogram of 0.07% DU solution dissolved in 0.01 M H_2SO_4. Chromatography conditions are detailed in Section 2.

[25,26]. The inhibitory activity of the LGP-free formulation was not documented after short-term incubation (Table 1). An increasing content of LGP improved antimicrobial protection of the product. The results of the challenge test suggest that the hydrogel should contain at least 0.1% of LGP in order to ensure antimicrobial protection of the product.

3.2. Development of the HPLC method

There were certain criteria during assay development, i.e. the method should be sufficiently sensitive, selective, and straightforward enough for routine use in the quality control laboratory, i.e. tedious sample preparation should be avoided. Also, the focus was made on measuring a true level of free formaldehyde in the formulation, which is hard to achieve by derivatization methods without prior separation of residual formaldehyde releaser.

The analytes of this study are very polar compounds and could not be retained on reversed phase columns without preliminary derivatization. As it follows from the chemical structures of diazolydinyl urea (Fig. 1) and propylene glycol, both compounds contains two or more alcoholic groups, though these groups are not present in the DU derivative after hydrolysis. From such structural analysis it was suggested that the exploitation of the HPLC column(s) designed for the separation of alcohols and sugars may provide suitable conditions for simultaneous analysis of both DU and PG. This type of columns has a stationary phase made of strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in either hydrogen or calcium form. The separation of the alcohols and sugars on this stationary phase is governed by the mixed mechanism of ligand exchange, size exclusion, ion exclusion and partition/adsorption. Since many of the analyzed carbohydrates generally contain keto- and aldehyde groups, possibility of the simultaneous analysis of DU, PG, and free FA was expected to be very probable.

As shown on the chromatograms of the standard solutions and hydrogel extract (Figs. 2 and 3) both FA and PG were eluted as narrow symmetric peaks with the retention time of about 9.4 and

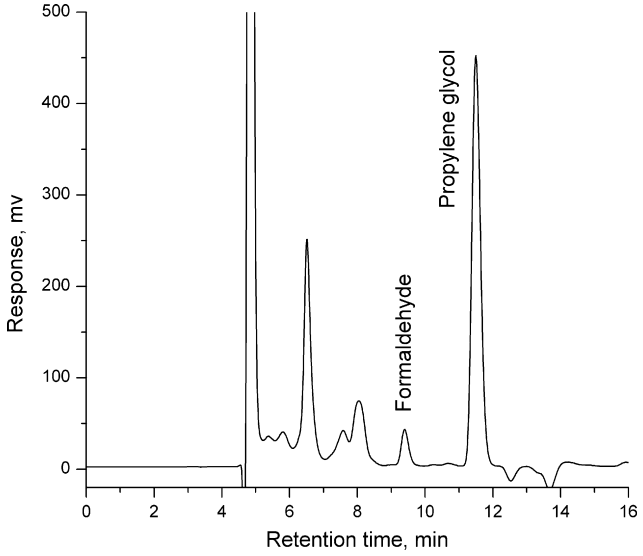

Fig. 3. Chromatogram of the extract of the hydrogel containing 0.5% LGP. Extraction by 0.01 M H_2SO_4. Chromatography conditions are detailed in Section 2.

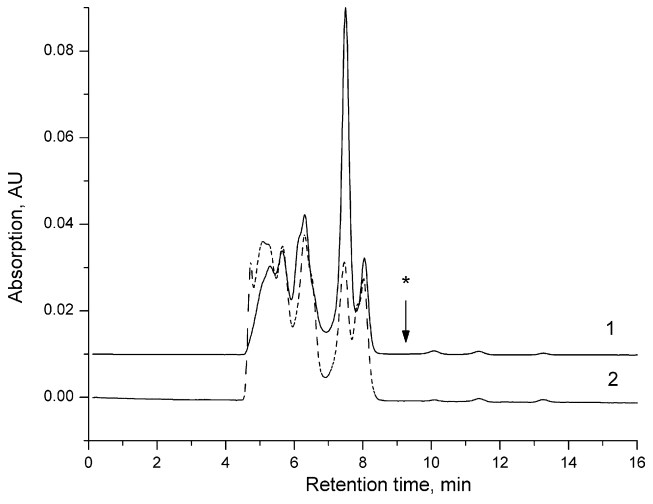

Fig. 5. Overlaid chromatograms of DU degraded in ammonium solution (1) and extract of hydrogel with 0.5% LGP obtained with PDA detection (λ – 210 nm). Arrow points out typical retention time of FA. Chromatography conditions are detailed in Section 2.

Table 2
Parameters of calibration curves used for determination of FA and PG in the hydrogel extracts.

Compound	Concentration range (μg/ml)	Range	Equation	r^2	SE intercept	SE slope	SD intercept
FA	35–280	Upper	$Y = 13021X - 14849$	0.99998	3424	21	5633
PG	125–1000		$Y = 13023X - 9938$	0.99999	8775	15	14434
FA	1–35	Lower	$Y = 12858X - 3528$	0.99997	870	36	1074
PG	5–125		$Y = 13064X - 2874$	0.99998	2475	31	3858

SE: standard error, SD: standard deviation of the mean.

11.5 min (N_{PG} = 10500, N_{FA} = 10100, T_{PG} = 1.20, T_{AF} = 1.18). To ensure a reproducibility of the retention characteristics and prevent absorption of positively charged molecules after hydrogel extraction, a diluted sulfuric acid (0.01 M) was used as a mobile phase. Moreover, acidic solvent used for the sample preparation ensured sufficient stability of residual DU, thus preserving an existing level of FA in the formulation. In chosen conditions relative standard deviation of the FA and PG areas in six replicate injections of standard preparations ranged from 0.1% to 0.7% proving good system precision.

3.3. Specificity

Given a non-specific sensitivity of the RI detector and a complexity of the retention mechanism of analytes on the ligand-exchange column, the method specificity was one of the primary concerns during the assay development. The selectivity of the method was verified by analyzing the chromatographs of the extracts of hydrogels, excipient, and potential degradation compounds. Chromatograms of the buffer solutions used for the hydrogel preparation and blank hydrogel extracts showed several large peaks near column void volume (4.8 min) while the critical one was completely resolved with formaldehyde ($Rs > 5$). Methanol, which is typically present in commercial formaldehyde as a stabilizer, eluted at 12.6 min and was completely resolved with PG ($Rs - 2.5$). IPBC, another component of LGP, gave no response on the chromatogram even at the maximal expected concentration.

Although earlier considered an individual compound, DU is now known to be a multi-component substance, with its major FA-releasing compound being 1-(3,4-bis-hydroxymethyl-2,5-dioxo-imidazolidin-4-yl)-1,3-bis-hydroxymethyl-urea (BHU) [24]. In aqueous solutions BHU decomposes with gradual release of formaldehyde and formation of HU as a final stable degradation product (Fig. 1) [24]. Besides BHU and products of its degradation, the presence of allantoin in the analyzed solutions cannot be ruled out, because allantoin is used as a starting component in the DU synthesis and may eventually contaminate commercial DU.

It was found that DU analyzed immediately after dissolution in the mobile phase gave small peak at 9.4 min, a set of peaks at 5–7 min and a major compound eluted at 7.6 min (Fig. 4). According to the retention time the peak eluting at 9.4 min could be attributed to FA, while no peaks were observed in the PG zone. It is also reasonable to suggest that the major component observed at 7.6 min is attributable to BHU, while the set of less retentive peaks includes HU, its intermediate degradation products or allantoin.

In order to exclude co-elution interferences, an ability of BHU, HU, and DU to absorb light in a far UV region due to 2,5-dioxo-4-imidazolidinyl fragment of the molecule was exploited. This property allows distinguishing DU and its degradation products from FA, which give no UV response even at highest analyzed concentration. Chromatogram of completely decomposed DU with the detection at 210 nm visualized a set of peaks in the 5–8 min region and revealed no signal at 9.3–9.4 min characteristic for the retention time of FA (Fig. 5). Similar HPLC pattern was observed for the extract of the hydrogel spiked with LGP, finally confirming that there was no co-elution of FA with BHU or its degradation products.

It is interesting to note, that allantoin itself elutes at 9.6 min and does not resolve with FA. However, being also a UV-absorbing compound allantoin was not observed in the detectable amounts among products of DU degradation. This indicates that allantoin is neither present in DU as an impurity nor appears during BHU decomposition, and supports the finding that HU is a final stable product of BHU degradation [24].

3.4. Stability of the analyzed solutions

Given a low stability of FA and its precursor DU in an aqueous solution, the stability of the extracts was verified in a short-term period. The extract of the LGP-containing hydrogel and the standard solutions of FA and DU were kept for 10 h at 5 °C and analyzed each hour in order to evaluate stability of the response. The standard solution of FA was stable for 10 h showing 0.4% RSD in the response. Both FA and PG were found stable in the extract of the LGP-laden hydrogel showing 1.2% and 0.1% RSD in the responses, respectively.

3.5. Linearity of response

Standard solutions of FA and PG were prepared in the concentrations corresponding approximately to 1–200% of their content in the hydrogel containing 0.5% of LGP. The level of FA was chosen as if all DU is hydrolyzed to form FA. The slope of the external standard plot, *y*-intercept and their standard errors are shown in Table 2.

Linearity was seen within the concentration range of 35–280 μg/ml and 125–1000 μg/ml for FA and PG, respectively. This upper concentration level covering a FA and PG concentration in all extracts was used for assay calculations.

In determining the quantification and detection limits, the slope and the standard deviation of the calibration curve in low concentration range were analyzed for each substance. The limit of

Table 3
Inter- and intra-day precision of FA and PG analysis in the hydrogel.

LGP content	RSD (%)					
	Day 1		Day 2		Overall	
	FA	PG	FA	PG	FA	PG
50% level (0.25% LGP)	0.80	0.73	0.71	0.62	2.34	0.67
100% level (0.50% LGP)	0.65	0.71	0.33	0.29	0.50	0.66
150% level (0.75% LGP)	0.37	0.80	0.31	0.26	1.06	0.88

Table 4
Recoveries of FA and PG after addition of known amount of LGP to the hydrogels containing different initial concentration of the preservative.

LGP content	FA		PG	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
50% level (0.25% LGP)	98.95	0.45	99.62	1.92
100% level (0.50% LGP)	100.21	0.41	100.50	0.25
150% level (0.75% LGP)	98.92	0.40	100.16	0.73

Table 5
Determination of FA and PG in the hydrogel dressings loaded with LGP by means of the HPLC method developed.

Hydrogel sample	FA		PG	
	Concentration (±SD) (%)	Recovery[c] (%)	Concentration (±SD) (%)	Recovery (%)
Lot 1 (0.25% LGP)[a]	0.0206 ± 0.0005	64.41	0.1517 ± 0.0010	101.16
Lot 2 (0.50% LGP)[a]	0.0382 ± 0.0002	59.63	0.3027 ± 0.0020	100.88
Lot 3 (0.75% LGP)[a]	0.0494 ± 0.0005	51.34	0.4477 ± 0.0039	99.50
Lot 4 (0.50% LGP)[b]	0.0302 ± 0.0005	46.74	0.3005 ± 0.0005	100.17
Lot 5 (0.50% LGP)[b]	0.0321 ± 0.0003	49.69	0.2992 ± 0.0012	99.73
Lot 6 (0.50% LGP)[b]	0.0304 ± 0.0004	47.01	0.3015 ± 0.0021	100.51
Lot 7 (0.50% LGP)[b]	0.0271 ± 0.0003	41.96	0.2986 ± 0.0012	99.53
Lot 8 (0.50% LGP)[b]	0.0272 ± 0.0005	42.09	0.2972 ± 0.0033	99.07

[a] Laboratory samples of hydrogel dressings.
[b] Pilot production batches.
[c] Recovery of FA was calculated assuming complete decomposition of DU to form FA.

detection (LOD) was defined as being a standard deviation of the intercept-to-slope ratio of at least 3:1 and the limit of quantification of at least 10:1. The LOD was 25 ng and 87 ng per injection for FA and PG, respectively. The LOQ was 84 ng and 291 ng per injection for FA and PG, respectively, which corresponded to 4.6×10^{-4}% (w/v) and 1.6×10^{-3}% (w/v) of the substances in the hydrogel. The lowest concentration of both FA and PG found in gel falls well above the LOQ established for these substances.

3.6. Intra-day and inter-day precision

FA and PG assay in hydrogel was performed in triplicate at 50%, 100%, and 150% concentration levels calculated to 0.5% of LGP for two different days. The precision of the assays on each day ranged from 0.31% to 0.80% and 0.26% to 0.80% for FA and PG, respectively (Table 3).

3.7. Accuracy

The accuracy of the procedure was demonstrated by the recovery studies, which were carried out by spiking the hydrogel samples containing 0.25%, 0.50%, and 0.75% of LGP with the standard solutions of FA and PG. Initial concentrations of FA and PG in the gels utilized in the calculations were taken from the precision study. As shown in Table 4, the recoveries were in the 98.92–100.50% range for both substances, illustrating good accuracy of the method.

3.8. Absolute FA and PG recovery

The results of the HPLC analysis of the prepared laboratory samples and pilot production batches of hydrogel formulations are shown in Table 5 in comparison with the theoretically calculated amounts of FA and PG from loaded LGP.

According to the results obtained, freshly prepared hydrogels contained an amount of PG very close to the theoretical values (99.1–101.2%) at all studied concentrations of LGP. The concentration of FA released reached steady level after several days following hydrogels preparation and corresponded to only 42–60% of maximum theoretical concentration that could be recovered after complete hydrolysis of DU in the hydrogel. Low recovery of FA in comparison with the theoretical level agrees well with the reported data showing that the overall degree of the FA recovery upon complete hydrolysis of DU reaches ca. 50% [27]. Therefore, we can conclude that the principal fraction of DU in the formulation at pH 5.5 at the moment of analysis has already decomposed, which is in line with the earlier observations of low stability of DU at pHs close to neutral [24]. The reasons of understated releasing properties of DU are beyond the scope of this article. The authors of paper [24] have suggested that such low level of available FA is due to contamination of commercial DU with HU and polymeric products of allantoin–formaldehyde condensation with lowered ability to generate FA and different releasing kinetics. In view of this fact, even the formulation containing 0.75% of LGP is not subjected to the labeling requirements (Table 5) and can be used to improve antimicrobial protection of the hydrogel formulation.

4. Conclusions

To the best of our knowledge this is a first report on the derivatization-free liquid chromatography method of FA analysis which also allows PG determination in a single run. The method is shown to be simple, precise and selective and allows determining a true level of FA in the presence of formaldehyde-releasing compounds. This analytical assay is a method of choice in developing new DU-containing formulations when the concentration of FA is a presumable subject of labeling requirements. The method might be applicable for various cosmetic, skin care and pharmaceutical formulations, though a selectivity of the FA and PG determination should be verified for each particular formulation.

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