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Short communication

Determination of urea, allantoin and lysine pyroglutamate in cosmetic samples by hydrophilic interaction chromatography

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Abstract

A new HPLC method using a Polyhydroxyethyl A column involving hydrophilic interaction chromatography (HILIC) is described for the simultaneous determination of urea, allantoin and lysine pyroglutamate in a cosmetic cream. Validation of the method was accomplished with respect to linearity, repeatability and limits of detection/quantification. Compound recoveries approach 100% with acceptable RSD values. The method is very simple since no derivatisation is necessary. Furthermore, it allows the rapid and direct chromatographic analysis of urea and hence could provide an alternative to other methods used to determine this compound in biological or cosmetic samples.

Keywords: Urea; Allantoin; Lysine pyroglutamate

1. Introduction

The hydration state of skin is affected by different mechanisms involving the superficial cellular layers, especially the intracellular hydrophilic natural moisturising factor (NMF), responsible for fixing water in epithelial cells [1]. Among the compounds usually employed, the sodium salt of pyrrolidone carboxylic acid (sodium pyroglutamate or sodium pidolate), a NMF component, is a rather good moisturiser. It may be combined with some amino acids such as lysine or arginine [2]. Urea is also traditionally used in dermatology for the treatment of dry skin. At low concentrations (<10%), it promotes water fixation on proteins [1]. The hydration action of these two molecules can be complemented by allantoin, used for skin protection and regeneration [3].

Most methods proposed for the determination of these compounds involve liquid chromatography (LC) or colorimetry. Pyrrolidone carboxylic acid can be directly determined by reversed-phase liquid chromatography (RPLC) [4,5] or after column pre-derivatisation allowing an improvement by the use of sensitive and selective fluorescence detection [6,7]. Other methods use a strong cation-exchange column [8]. Urea is mainly analysed using colorimetric and enzymatic methods [9,10]. New methods involving high-performance thin layer chromatography (HPTLC)–densitometry and colorimetry have also

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been described [11]. Spectrophotometry, alkalimetric titration and chromatography allow allantoin determination in biological, cosmetic and pharmaceutical samples [3]. LC is the most specific method and mainly uses C_{18} hydrophobic columns. However, allantoin is poorly retained and must be detected at short wavelengths, conditions that favour interference from coeluting compounds [3]. Ion pairing chromatography can be a good alternative [12]. A pre-column derivatisation converting allantoin to a phenylhydrazone absorbing in the near UV range can be used to increase retention and improve detection [13,14]. A method using micellar electrokinetic capillary chromatography [15] is also described.

These hydrophilic molecules should be retained under normal-phase chromatographic (NPC) conditions but they do not dissolve well in the non-aqueous mobile phases typically used in NPC. One solution could be the use of polar stationary phases like uncoated silica, diol- or amino- phases [16,17] eluted with mixed aqueous–organic mobile phases. This mode of chromatography has been termed as Hydrophilic Interaction Chromatography (HILIC) by Alpert in 1990 [18]. HILIC is characterised by the presence of a high initial organic modifier concentration to favour hydrophilic interactions between solutes and the hydrophilic stationary phase. It is particularly well suited for the separation of polar biological solutes like proteins, peptides, amino acids and carbohydrates [19,20] and special HILIC columns have been designed for such separations [18] (e.g. poly(2-hydroxyethylaspartamide)-silica). This very polar phase allows a decrease in the organic solvent level necessary to get retention, making possible the determination of many compounds of marginal solubility in a predominantly organic solvent. Mixed-mode hydrophilic interaction–cation-exchange chromatography (HILIC–CEX) promotes hydrophilic interactions overlaid on ionic interactions with the cation-exchange matrix and this novel high-performance technique has an excellent potential for peptide separations [21,22].

In this paper, we propose a new method which allows isocratic simultaneous separation of urea, allantoin and lysine pyroglutamate (lysidone) in a cosmetic cream by HILIC chromatography using a Polyhydroxyethyl A column.

2. Experimental

2.1. Reagents

HPLC-grade water and acetonitrile (ACN) were obtained from Baker (Deventer, The Netherlands). ACS grade orthophosphoric acid (PA), sodium dihydrogen phosphate (SDHP), sodium acetate (SA) and hexane were obtained from Merck (Darmstadt, Germany) and triethylamine (TEA) from Lancaster (Morecambe, UK). Celite (infusorial earth) was obtained from Merck Eurolab (Lyon, France). Standards of urea and allantoin were purchased from Merck and lysidone from UCIB (Anet, France).

2.2. Instrumentation

The HPLC system consisted of a SpectraSystem P1000 pump, a 20 μl Rheodyne model 7125 injector and a SpectraSeries UV150 detector coupled with a SP4600 Data Jet integrator from ThermoQuest (San Jose, CA, USA).

2.3. Column and chromatographic conditions

Separation was achieved using a 5 μm, 60-Å pores Polyhydroxyethyl A column (200×4.6 mm I.D.) (PolyLC, Columbia, MD, USA). The mobile phase was triethylamine phosphate (TEAP), pH 2.8, containing 80% ACN (v/v), the final concentration of TEAP being 6 m*M* overall. The TEAP solution was a dilution of a concentrated pH 2.8 stock solution prepared fresh monthly. The stock solution of TEAP was prepared by making a concentrated aqueous solution of PA, adding TEA until the desired pH was attained, then diluting to give a 2 *M* final concentration in phosphate. Appropriate amounts of stock solution, water and ACN were mixed together for preparation of the mobile phase, which was then filtered and degassed under vacuum by passing through a 0.45 μm nylon filter. Before a series of injections, it was necessary to condition the column for 1 h with a 0.02 *M* SDHP+0.03 *M* SA buffer solution in the pH range of 3–6, flush with 20 ml of water and equilibrate at least 2 h with the mobile phase. This conditioning is related to the mechanism of HILIC, which involves a partitioning between the

mostly organic mobile phase and a stagnant layer of water on the surface of the stationary phase [18]. The role of the SDHP/SA and water washes is to regenerate the surface aqueous layer in a reproducible fashion so as to obtain stable retention times and analyte separation. Chromatography was achieved at ambient temperature under isocratic conditions at a flow-rate of 1 ml/min with a run time of less than 10 min. Eluted mobile phase was monitored at 200 nm and compounds were identified by the retention times of standards. Sample injection volume was 20 μl.

2.4. Standard solutions

A stock standard solution was prepared by dissolving urea (0.2 g), allantoin (0.02 g) and lysidone (0.6 g) in 20 ml of water. The working standard solution was obtained by diluting stock solution (1/100) with mobile phase. For linearity studies, the stock solution was diluted as appropriate with mobile phase to obtain concentrations ranging from 60 to 140% of the working standard solution concentration.

2.5. Sample preparation

Cosmetic cream (0.5 g) was mixed with celite (0.5 g) and extracted three times with hexane (10 ml). Organic phases were discarded and the residue was dried at 60°C. Addition of water (3 ml) was followed by mechanical shaking. This operation was repeated three times. Water extracts were combined in a 10 ml glass flask and water was added up to 10 ml. After mixing, the solution was successively filtered through 0.45 μm and 0.2 μm nylon filters. It was then diluted with ACN (1:5) and filtered again before injecting in duplicate onto the column.

3. Results and discussion

Fig. 1 shows working standard and sample solution chromatograms. No interference occurs with the other sample components, unretained on the Polyhydroxyethyl A column. Mean retention times (±SD) for urea, allantoin and lysidone are 5.33 min (±0.10), 5.92 min (±0.09) and 7.01 min (±0.12), respectively based on 40 calibration standard injections. In spite of close retention times between urea and allantoin, the resolution (Rs≈1.5) allows accurate quantitative determination.

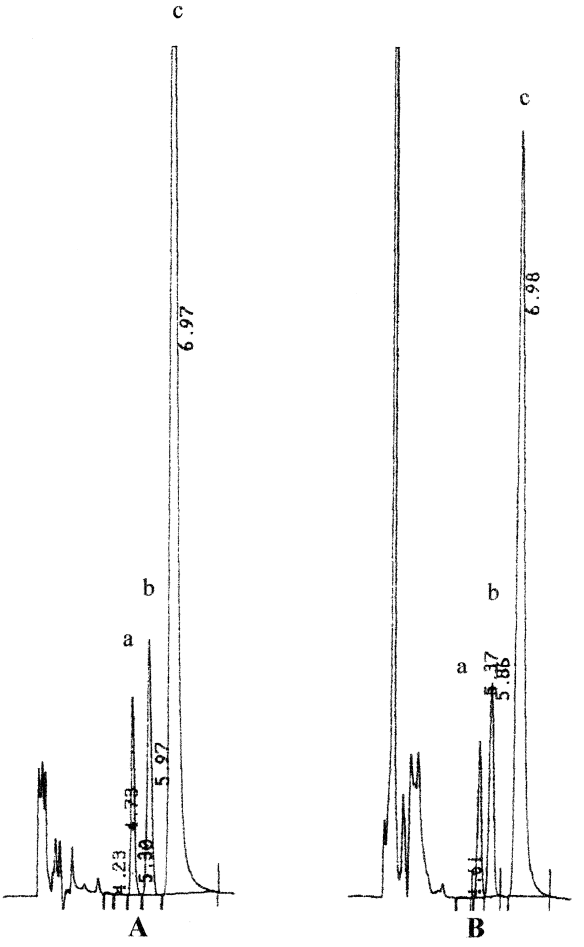

Fig. 1. Working standard solution (A) and sample solution (B) chromatograms. Peaks: a=urea, b=allantoin, c=lysidone.

During method development, the effects of mobile phase composition on compound retention were evaluated and a graph of analyte retention times vs. % ACN is presented in Fig. 2. Increasing the proportion of ACN in the mobile phase increased all retention times, with allantoin now eluting after lysidone. Decreasing the ACN proportion decreased all retention times with co-eluting peaks for urea and allantoin. Increasing TEAP up to 10 m*M* in the mobile phase decreased all retention times. Allantoin

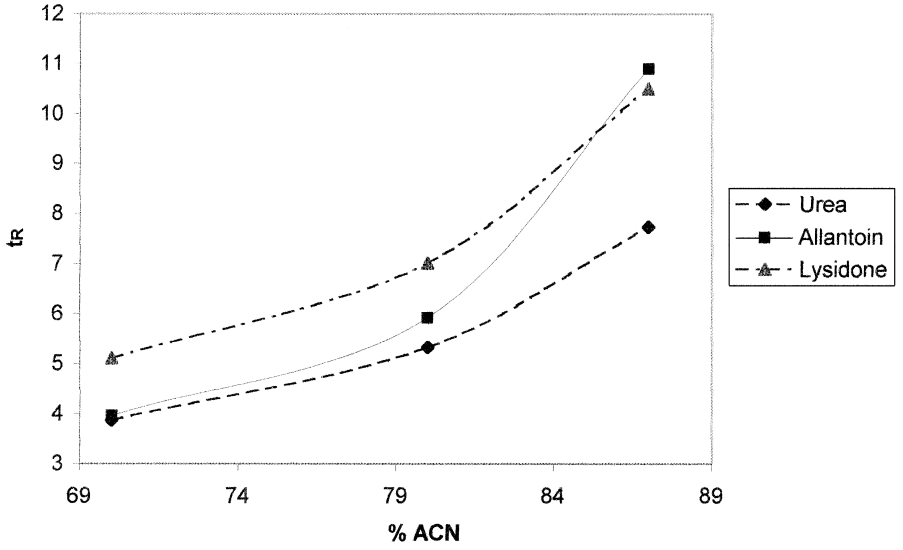

Fig. 2. Influence of mobile phase composition on retention times.

and lysidone were co-eluted and a poor separation from urea was observed. Decreasing TEAP down to 2 m*M* made retention times unstable, especially for lysidone.

First, the repeatability of the injection was checked by performing six replicate injections of the working standard solution. The relative standard deviation (RSD %) was $<2\%$ for all products.

In order to evaluate the linearity of the method, calibration curves were made by performing triplicate injections at five standard concentrations for each compound. Peak area responses to each compound concentration were highly linear with regression coefficients above 0.995 over the range of concentrations tested. Regression equations are listed in Table 1. A high degree of significance was obtained for the statistical tests carried out from regression analysis. In each case, the intercept value was not significantly different from zero ($\alpha=0.05$). Limits of detection (LOD) and limits of quantification (LOQ) at $P=95\%$ level of significance were calculated by a statistical treatment of calibration data [23] and are presented in Table 1.

Compound recoveries for cosmetic cream samples were calculated by comparison of peak area responses determined for the calibration standards with those determined for diluted cream extracts. The observed values (Table 2) are in good agreement with the expected ones. The relatively high value obtained for the RSD is related to the extraction step used to remove the water insoluble matrix from the cream. The addition of a suitable internal standard (I.S.) to the cream before extraction to overcome this problem was not possible. However, the inability to use an I.S. was not considered a major disadvantage since recoveries of all compounds are close to 100%

Table 1
Linearity (regression analysis), limit of detection (LOD) and limit of quantification (LOQ) of urea, allantoin and lysidone

Compound	Concentration range (μg/ml)	Equation[a]	Correlation coefficient	LOD (μg/ml)	LOQ (μg/ml)
Urea	60–140	$y=5048(\pm103)x-5042(\pm10961)$	0.999	7.5	22.7
Allantoin	6–14	$y=86598(\pm1294)x-18507(\pm13717)$	0.999	0.7	1.8
Lysidone	180–420	$y=17424(\pm199)x-46098(\pm62934)$	0.999	13.5	38.8

[a] Linear regression equation with $x=$compound concentration (μg/ml) and $y=$peak area.

Table 2
Recoveries of urea, allantoin and lysidone in the cream sample

Compound	Number of samples tested (*n*)	Expected value (g/100 g)	Observed value (g/100 g)	Recovery (%)[a]	RSD (%)[b]
Urea	9	1.000	0.998	99.8	6.7
Allantoin	9	0.100	0.097	97.1	8.2
Lysidone	9	3.000	2.886	96.2	5.9

[a] Recovery was calculated as: [(Expected value − Observed value)×100]/Expected value.
[b] RSD = relative standard deviation.

and RSD values are in the same order of magnitude as those described in other papers involving allantoin determination in biological fluids or active compounds in cosmetic samples [12,13,15].

Before using the Polyhydroxyethyl A column, separation of allantoin and lysidone was performed on a 10 μm Nucleosil C_{18} column (250×4.6 mm I.D.) (data not shown). A good resolution was obtained with a mobile phase of methanol/phosphate buffer 0.05 *M*+0.5% TEAP pH 6.3 (3:97, v/v), but the two compounds (t_R allantoin=2.54 min, t_R lysidone=3.17 min) were co-eluted with some sample excipients near the column dead time ($t_0 \approx 2$ min). Under these conditions, urea was eluted at the dead time.

Other polar stationary phases such as cyano- or amino- were not tested owing to the satisfactory results obtained with the Polyhydroxyethyl A column. The only drawback is the nonreproducibility of retention times without column conditioning as indicated in the experimental section.

4. Conclusion

Hydrophilic interaction chromatography (HILIC), which has recently been introduced as a potentially useful separation mode for the determination of peptides and other polar compounds, could be successfully applied to the simultaneous determination of urea, allantoin and lysine pyroglutamate in a cosmetic cream. Validation of the method was accomplished with respect to linearity, repeatability and limits of detection/quantification. Compound recoveries approached 100% with acceptable RSD values and the method is very simple since no derivatisation is necessary. Furthermore, because urea is an important compound in biology and is widely used in cosmetics, the present HPLC method has the advantage of being simple, without handling and time consuming reaction steps when compared to other methods mainly involving colorimetry. It allows a rapid, specific, reproducible and sensitive determination of this compound in complex mixtures and consequently could be useful in the analysis of biological samples for diagnostic purposes.

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