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Analytical methods for measuring urea in pharmaceutical formulations

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Abstract

Two new methods are described for the routine determination of urea that utilize HPTLC-densitometry and colorimetry. The methods involve derivatization of urea with *p*-dimethylaminobenzaldehyde to a yellow-coloured compound. Validation of the methods was accomplished with respect to linearity, accuracy, reproducibility and limit of detection/quantification. Both methods were compared with an enzymatic method previously described in the literature and were found to be in close agreement. The proposed methods have the advantages of being simple, rapid and involve a single step sample preparation. Under experimental conditions HPTLC was the most sensitive method.

Keywords: Urea; Analytical methods; HPTLC; Densitometry; Colorimetry

1. Introduction

The use of urea in dermatological products has increased significantly in the last few years because of its many effects on human skin. The dermatopharmacological properties of urea were recently reviewed in [1–4].

Several methods have been described for the quantification of urea [5–14]. Most of these methods are based either on the derivatization of urea to a coloured product [5–10] or enzymatic hydrolysis by urease to ammonia and carbon dioxide followed by measurement of the ammonium ion concentration [11–14]. Among the various methods published for the direct colorimetric determination of urea, the most investigated have been those based on the Fearon [5] carbamido reaction between the urea compound and diacetyl monoxime or diacetyl [6–12]. However, the original Feron method has many problems such as reactions between constituents of the chromogenic reagent, prolonged boiling time, insufficient detection limit, instability of the colour to light, and a non-linear calibration curve. Consequently, the reaction conditions have been investigated and various improvements have been proposed in the literature [6–9]. Although the urease procedures are accurate and convenient means of assaying urea, they are time consuming and, therefore, unsuitable for serial estimation of urea in a large

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number of samples. Therefore, there is an increasing demand concerning efficient and rapid analytical assays to support the pharmaceutical and biopharmaceutical characterization of topical urea formulations during process development, optimization and scale-up production.

In view of the above shortcomings, two new methods for the determination of urea that utilize colorimetry and HPTLC-densitometry have been developed. The colorimetric method was successfully applied in routine characterization of semisolid urea formulations and in vitro release experiments.

2. Experimental

2.1. Materials and reagents

The urea reference substance, absolute ethanol, sulphuric acid and HPTLC plates, precoated with silica gel 60 (10×10 cm) were obtained from Merck (Darmstadt, Germany). *p*-Dimethylaminobenzaldehyde was obtained from Sigma (Deisenhofen, Germany). Test-combination for determinations of urea–ammonia Cat.No. 542946 was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals and solvents were of analytical grade of purity.

2.2. Spectrophotometric method

A solution (0.5 ml) containing 4% (w/v) of *p*-dimethylaminobenzaldehyde and 4% (v/v) sulphuric acid in absolute ethanol was added to 2 ml of a solution of urea. After 10 min, the absorbance of the solution was measured at 422 nm against a reagent blank using a Shimadzu spectrophotometer (Duisburg, Germany). The concentrations of the yellow-coloured compound in the samples were determined by reference to the calibration curve. The absorption spectra were recorded against a reagent blank using a Hewlett-Packard 8452A Diode Array spectrophotometer.

2.3. HPTLC-densitometric method

A volume of 10 μl urea solution was applied to a HPTLC plate in a 5 mm strip, 12 mm from the lower edge of the plate using a TLC-applicator AS 30 Desaga (Heidelberg, Germany). The plate was developed at room temperature in an unlined glass tank (Desaga) containing absolute ethanol and 13.5 M ammonia (99:1, v/v) (according to British Pharmacopoeia, 1993 [15]). The mobile phase was allowed to run a distance of 80 mm. After solvent evaporation, the plate was sprayed with a solution containing 0.5% (w/v) of *p*-dimethylaminobenzaldehyde and 0.5% (v/v) of sulphuric acid in absolute ethanol by using a sprayer SG1 (Desaga, Germany). The Rf value of urea derivatization product was 0.54. After standing the plates for 10 min in the open air, the peak area of each spot was measured by densitometry. To determine the concentration of unknown samples, three different standard concentrations (10, 20, and 50 μg ml^{-1}) were also applied so that a calibration graph was included for each plate.

Quantification of the thin-layer chromatograms was carried out with a Desaga CD60 densitometer (Heidelberg, Germany) by linear scanning in the remission-extinction mode at 418 nm. The analysis conditions were: band width, 0.2 mm; band length, 2 mm; swing width, 0.5 mm. Concentrations were measured by calculating the peak areas of the standards and the samples.

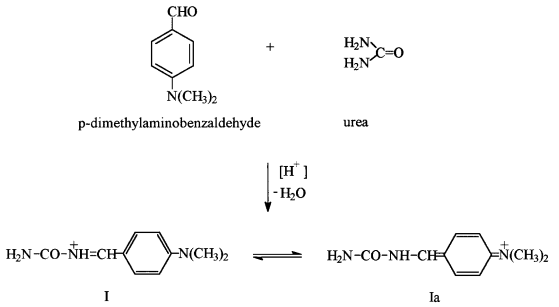
2.4. Determination of urea in ointment

4 ml Absolute ethanol were added to 0.1 g of ointment. The mixture was heated on a water bath until dispersed and placed for 30 min on a mechanical shaker. The sample was then diluted to 50 ml with absolute ethanol and filtered through a 0.2 μm filter (Chromafilm® Macherey-Nagel, Germany). 5 ml Of the filtrate was diluted with the same solvent to 50 ml and aliquots of 2 ml and 10 μl were taken and treated as described above for colorimetric and HPTLC measurements, respectively.

3. Results and discussion

The starting-point of the two developed analytical methods is the derivatization of urea with *p*-dimethylaminobenzaldehyde to a yellow-coloured compound [12,13]. According to the literature [13] the yellow product might be a simple Schiff base with quinoid resonating structure (Scheme 1). The absorption spectra of the coloured compound obtained by the colorimetric method proposed may be seen in Fig. 1. This method has the advantages of being a direct and rapid analytical method, does not require heating for the reaction to take place and produces a colour stable derivative. The colour develops rapidly and reaches a fairly steady value in less than 5 min and remains stable for at least 30 min (Fig. 2). The linearity of the calibration graphs, constructed in the concentration range from 8 to 128 μg ml^{-1}, is demonstrated by the high determination coefficients ($r^2 > 0.999$; $n = 5$). Beer's law was given by the equation: $y = 0.00721 + 0.00715x$; $r = 0.9999$. The S.D. of residuals from the line ($S_{y.x}$) $= 5.19 \times 10^{-3}$. The intercept value was not significantly different from zero ($P < 0.05$).

For the high-performance thin-layer chromatography method the derivatization reaction was performed after development of the chromatograms by spraying with the chromogenic reagent. Homogeneity of spots and uniformity of the background are the factors that permit this reagent to be used for densitometric evaluation of urea reaction product. All the calibration graphs obtained showed good linearity in the concentration range from 0.1 to 0.5 μg ($r^2 > 0.99$; $n = 5$). For the regression equation $y = 2419.5x + 63.2$, where x is the amount of urea (μg) and y is the peak area, the correlation coefficient was 0.999. The S.D. of residuals from the line ($S_{y.x}$) $= 10.55$.



Scheme 1. The structure of the yellow product.

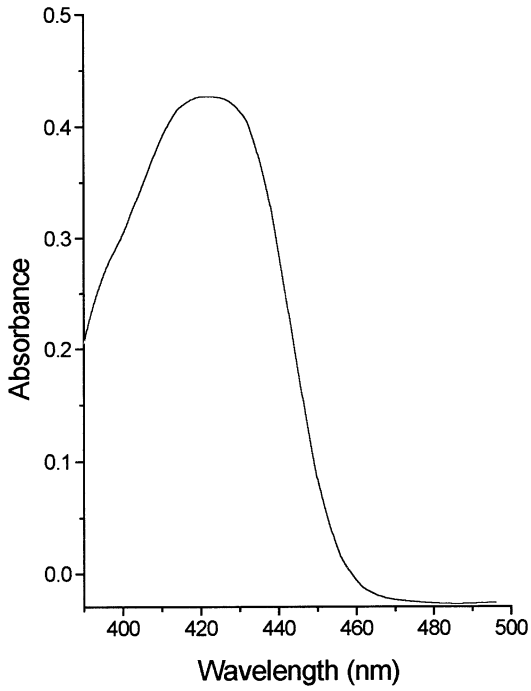


Fig. 1. Absorption spectra for the reaction product of 64 μg ml^{-1} urea with *p*-dimethylaminobenzaldehyde solution. (See Section 2 for experimental conditions).

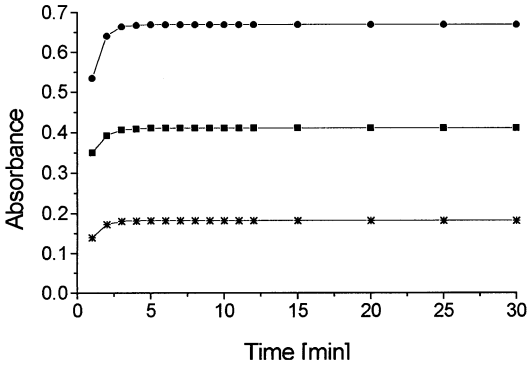


Fig. 2. Colour development of urea derivatization compound at 422 nm. (●-●) 93 μg ml^{-1}, (■-■) 56 μg ml^{-1} and (∗-∗) 25 μg ml^{-1} urea with *p*-dimethylaminobenzaldehyde solution. (See Section 2 for experimental conditions).

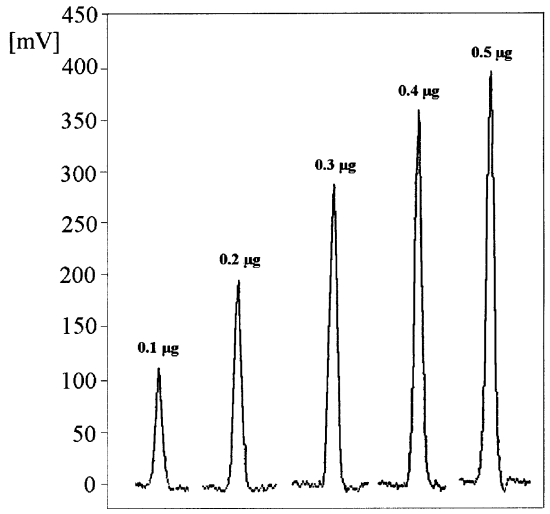


Fig. 3. Typical densitogram for quantitative evaluation of urea derivatization compound. (See Section 2 for chromatographic conditions).

The HPTLC method showed a statistical difference from zero ($P < 0.05$) in the intercept values. A typical densitogram for quantitative evaluation of urea is presented in Fig. 3. The stability of the spots after colour development was measured by scanning the same plate (containing standard solutions in the range from 0.1 to 0.5 μg) six times consecutively over a period of 2 h. The coefficient of variation (%) for peak area obtained ranged from 0.2 to 0.6% indicating the good stability of derivatization product during the scanning procedure. After this period the intensity of the colour reaction diminishes significantly ($P < 0.05$), but the linear relationship between concentration of the urea reaction product and spot area remains unchanged. The specificity of the HPTLC assay is guaranteed by the absence of interfering peaks due to the excipients.

Comparisons were made under routine conditions between the colorimetric and HPTLC methods developed and one spectrophotometric method employing urease [14]. The parameters analysed are shown in Tables 1–3.

Limits of detection and limits of quantification at a $P = 95\%$ level of significance, calculated by a statistical treatment of calibration data (standard solutions) [17], were respectively 5.18 and 7.63 μg ml^{-1} for the colorimetric method, and 1.87 μg ml^{-1} (56 ng spot^{-1}) and 2.77 μg ml^{-1} (83 ng spot^{-1}) for the HPTLC method (Table 1). These values of quantification limits are lower to that obtained by the enzymatic method previously described in the literature [14] (Table 1). The repeatability of the different methods were checked by performing six replicate determinations of a standard solution containing 20 μg ml^{-1} urea. For the HPTLC method the repeatability was determined by measuring the peak areas obtained from replicate ($n = 6$) of urea derivatization product on the same chromatographic plate.The coefficient of variation (%) was 2.41% for the urease procedure and 3.84 and 2.72% for the HPTLC and colorimetric methods, respectively (Table 2).

Accuracy experiments were carried out by incorporating different amounts of standard urea into an O/W emulsion (9, 10 and 11%; Wasserhaltige hydrophile Salbe DAB 10) [16]).

Table 1
Limit of detection/quantification according to different methods of determination

Analytical method	Detection limit (μg ml^{-1})	Quantification limit (μg ml^{-1})
HPTLC[a]	1.87 (56 ng spot^{-1})	2.77 (83 ng spot^{-1})
Colorimetry	5.18	7.63
Urease[b]	n.m[c]	10[b]

[a] Amount applied on the chromatographic plate: 30 μl (standard solutions ranging from 0.1 to 0.5 μg). Other conditions as described in the text.
[b] According to [14].
[c] n.m., Not measured.

Table 2
Repeatability ($n = 6$) according to different methods of determination

Analytical method	Concentration (μg ml^{-1})	Repeatability (CV%)
HPTLC	20	3.84
Colorimetry	20	2.72
Urease[a]	20	2.41

[a] According to [14].

Table 3
Recovery data for different content levels of urea in the ointment

Analytical method	Amount of urea added (%)	Amount found (%) mean ± S.D. ($n = 6$)	Recovery (%)
HPTLC	9.0	9.17 ± 0.32	101.89
	10.0	10.36 ± 0.32	103.60
	11.0	11.41 ± 0.39	103.72
Colorimetry	9.0	8.98 ± 0.13	99.83
	10.0	10.10 ± 0.22	99.01
	11.0	10.99 ± 0.26	99.88
Urease	9.0	9.00 ± 0.26	100.15
	10.0	10.18 ± 0.37	101.80
	11.0	11.11 ± 0.43	101.03

The results of the accuracy studies are given in Table 3. Mean recovery values of 103.07% for the HPTLC method and 99.57% for the colorimetric method were obtained (means of three different concentrations). Statistical analysis of the results using the *t*-test for paired data revealed no significant differences between the two developed methods and the urease method ($P < 0.05$).

4. Conclusions

The analytical methods presented showed good agreement with the enzymatic method and are reliable and effective for the quantification of urea from topically applied products. Compared with the enzymatic method, the proposed procedures have the advantages of being simple, rapid and without handling and time-consuming reaction steps. Thus, a large number of samples can be analysed within a short time. In addition the quantification limits obtained by the proposed methods were lower to that obtained by the enzymatic method. Under experimental conditions the HPTLC method was the most sensitive method.

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