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Simultaneous determination of ketoconazole and formaldehyde in a shampoo: liquid chromatography method development and validation

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Abstract

Ketoconazole is an antifungal agent, which is the active ingredient in a shampoo primarily used for the treatment of seborrhatic dermatitis (anti-dandruff shampoo). The shampoo also contains imidazolidinylurea as a formaldehyde releasing preservative. The aim of this study was to develop a HPLC system that allows the determination of both ketoconazole and formaldehyde. The finally selected isocratic system consisted of an Interchrom Nucleosil (250×4.6 mm, 5 μm) C_8 column and a mobile phase containing acetonitrile–phosphate buffer 0.025 *M*, pH 4.0, 45/55 (v/v). Ketoconazole could immediately be determined at 250 nm after injection of diluted shampoo. Formaldehyde was measured at 345 nm after derivatisation with a 2,4-dinitrophenylhydrazine solution. At the selected conditions, the other excipients of the shampoo did not interfere in the assays for both substances. Method validation was performed on both assays. Different selectivity towards ketoconazole and formaldehyde was observed when applying other C_8 columns. This fact, however, did not affect the assays of both substances.

Keywords: Shampoo; Method development; Validation; Selectivity; Ketoconazole; Formaldehyde

1. Introduction

Ketoconazole is an antifungal agent that is administered topically or orally. It is an imidazole derivative [1] with the structure shown in Fig. 1. Ketoconazole is the active ingredient in the commercialized antifungal shampoo Nizoral® (Janssen Pharmaceutica) that is primarily used for the treatment of seborrhatic dermatitis (anti-dandruff shampoo). Actually, ketoconazole is commercially available as bulk product, which allows the development of generic anti-dandruff shampoos. In this paper the determination of ketoconazole was performed in such a generic shampoo preparation.

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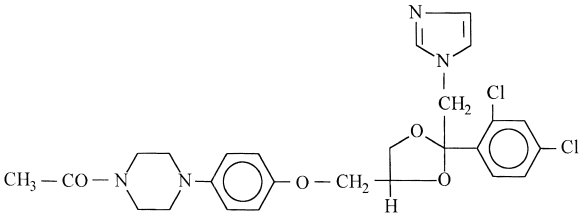

Fig. 1. Structure of ketoconazole.

This latter shampoo also contained imidazolidinylurea (imidureum) (Fig. 2) as a formaldehyde releasing preservative to prevent microbial contamination. However, even though formaldehyde or formaldehyde releasing preservatives are frequently used in cosmetic preparations such as shampoos and skin-care products, it is an irritant agent which can cause allergic contact dermatitis or formaldehyde-sensitive eczema [2–5]. Therefore European and FDA (Food and Drug Administration) regulations exist which regulate the maximum free formaldehyde content [3,5–7]. The use of formaldehyde as a preservative in cosmetic products, e.g. cosmetic hair products, is allowed up to a maximum concentration of 0.2% (with the exception of nail hardeners for which a concentration up to 5% is allowed), but if the concentration exceeds 0.05% the product has to be labeled ''contains formaldehyde'' [3,5,6,8]. For this reason a formaldehyde assay is required. The official EC method is based on the condensation of free formaldehyde with ammonium acetate and acetylacetone to form fluorescent 3,5-diacetyl-1,4-dihydrolutidine which is selectively detectable [9,10]. However, this method is not suitable when formaldehyde donors are present in the cosmetic formulation because additional formaldehyde is released during the assay [9]. For cosmetics containing formaldehyde donors the official EC method of Ref. [10] was updated [11]. The assay for free formaldehyde of Ref. [11] considers a HPLC separation of formaldehyde from the other compounds followed by a post-column derivatisation using the above-mentioned reaction. The HPLC system consists of a C_{18} column and a 100% aqueous mobile phase (phosphate buffer, pH 2.1). A colorimetric method to determine free formaldehyde, released from formaldehyde donors, in anionic shampoos is described in Ref. [12]. More recently a flow injection method was proposed [7], while also high-performance liquid chromatography (HPLC) methods were developed [4,9]. These methods, using pre-column derivatisation with 2,4-dinitrophenylhydrazine (2,4-DNPH), allow to assay free formaldehyde in the presence of its donor [9]. A chromatographic method to separate imidazolidinylurea from a number of other components in a commercial cosmetic cream has been described in Ref. [13].

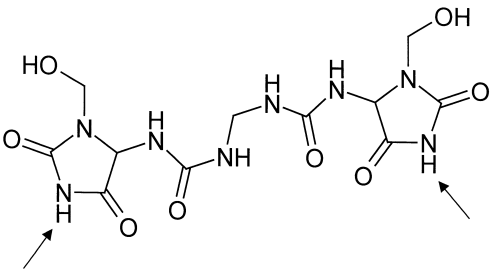

Fig. 2. Structure of imidazolidinylurea.

For the analysis of ketoconazole in pharmaceutical dosage forms, spectrophotometric and spectrofluorimetric methods are described [14], while HPLC methods are reported for the analysis in plasma and organs [15], as well as in pharmaceutical preparations such as tablets, creams and shampoos [16]. However, in none of these methods ketoconazole and formaldehyde are determined using the same HPLC system.

The aim of this study was to develop one HPLC system, i.e. a stationary and mobile phase combination, that allows determination of both ketoconazole and formaldehyde. The detection used was UV–Vis absorbance. Ketoconazole is a UV-absorbing substance while formaldehyde is transformed to a UV-absorbing compound in a pre-column derivatisation reaction with 2,4-dinitrophenylhydrazine [9]. Initially our intention was to develop a method that also allowed assay of imidazolidinylurea. For reasons discussed further this idea was later abandoned. The developed methods were then subjected to method validation. The validation characteristics evaluated were the selectivity towards the other excipients in the shampoo, the precision, the linearity range, the bias, the detection limit (if relevant) and the robustness [17–32].

2. Experimental

2.1. Chemicals

Ketoconazole was obtained from Erregierre SPA (S. Paolo D'argon, Italy) and from Kraemer & Martin (St. Augustin–Buisdorf, Germany); imidurea, imidazolidinylurea or Germall 115 from ISP (St. Niklaas, Belgium), sodium laurylether sulphate or LES 28 (NaLES) as a 28% aqueous solution from Eur-O-Compound (Oudenaarde, Belgium), disodium laurylether sulfosuccinate or Euranaat LS3 (Na_2LESS) from Eur-O-Compound, Comperlan KD or coconut fatty acids diethanolamide from Henkel (Düsseldorf, Germany), macrogol 120 methylglucose dioleate or Glucamate DOE-120 from Amerchol (Edison, NJ, USA), sodium chloride (NaCl) from Merck (Darmstadt, Germany), Orange-Yellow S or Sunset Yellow FCF from BASF (Ludwigshafen, Germany) and hydrochloric acid (HCl) 1 *M* from Merck.

Formaldehyde 37% m/m solution was obtained from Merck, acetonitrile from BDH Supplies (Poole, UK), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), 2,4-dinitrophenylhydrazin (2,4-DNPH), H_3PO_4 85%, HCl 32% and NaOH 1 *M* solution, all were of pro analysis (GR) grade and were supplied by Merck. Water for preparation of buffer and reagent solutions was produced in-house by the Milli-Q water purification system (Millipore, Milford, MA, USA). All buffer solutions were filtered through a 0.2-μm membrane filter from Schleicher & Schuell (Dassel, Germany). The mobile phase was degassed in an ultrasonic bath before use.

2.2. Composition of shampoo

The examined shampoo contained 2% ketoconazole, 0.3% imidurea, and further NaLES 28% solution, Na_2LESS, Comperlan KD, Glucamate DOE-120, NaCl, Orange-Yellow S, HCl 1 *M* till pH 6.5 and purified water till 100%. The percent values mentioned above are m/m% values.

2.3. Apparatus

The chromatographic system used consisted of a Merck-Hitachi L-6000 Intelligent Pump (Tokyo, Japan) equipped with a Rheodyne (Cotati, CA) injector, a Perkin-Elmer LC90 UV Detector (Shelton, CT) and a Merck-Hitachi D-2500 Chromato-Integrator. The column used was an Interchrom Nucleosil C_8, 250×4.6 mm I.D., 5 μm (Interchim, Montluçon, France)

2.4. Chromatographic conditions

The mobile phase contains a mixture 45/55 (v/v) of acetonitrile and 0.025 *M* $NaH_2PO_4.H_2O$ aqueous solution, adjusted to pH 4.0 with H_3PO_4 1 *M* solution. At nominal conditions, analyses were performed at a flow-rate of 1 ml/min, at room temperature and at detection wavelengths of 250 nm for ketoconazole and 345 nm for formaldehyde. The injection volume was 20 μl.

2.5. Standard and sample solutions

Stock solutions of 1 mg/ml of ketoconazole and 0.1% (m/v) of formaldehyde were prepared in the mobile phase (27.0 ml of 37% formaldehyde solution was diluted to 100.0 ml with mobile phase to obtain a 10% intermediate stock solution). Since formaldehyde is volatile, the exact concentration of formaldehyde 37% standard solution was determined prior to analysis. The assay for formaldehyde is described in the European Pharmacopoeia [33]. Formaldehyde reacts with iodine solution and the excess of iodine is back-titrated with sodium thiosulphate using starch as indicator. The concentration of sodium thiosulphate was determined using the reaction with potassium bromate, and the iodine solution was standardised with sodium thiosulphate solution, according to the procedures described in Ref. [33]. The concentration of the formaldehyde solution used was found to be 37.33%.

Working solutions of ketoconazole and formaldehyde were obtained by diluting the stock standard solution with mobile phase. The dilutions of shampoo were prepared using the following scheme: accurately weigh 1.0 g of shampoo (in a volumetric flask containing already some mobile phase) and dilute to 10.0 ml with mobile phase (=shampoo stock solution). The final dilution was then obtained

by further diluting this stock solution. Samples and standards were prepared daily prior to injection.

For formaldehyde, the reaction with 2,4-DNPH of Ref. [9] was used. In this derivatisation 0.4 ml of 2,4-DNPH 0.1% solution is added to 1.0 ml sample or standard. The mixture is vortexed for 1 min and allowed to stand at room temperature during 2 min. The solution is then stabilised by adding 0.4 ml of a phosphate buffer 0.1 *M* (pH 6.8) and 0.7 ml of NaOH 1 *M*. This mixture is then injected onto the column.

The reagent solution 2,4-DNPH was prepared in a mixture 40:60 (v/v) of HCl 32% and water. The phosphate buffer pH 6.8 was an aqueous 0.1 *M* NaH_2PO_4 solution adjusted to pH by NaOH 1 *M*.

3. Results and discussion

3.1. Method development

The composition of the shampoo was developed in-house [34]. In a first instance, we tried to develop a HPLC method that would be capable of determining imidurea, formaldehyde and ketoconazole in one run or using one chromatographic system. Therefore experiments were started from the conditions described by Sottofattori et al. [13] which were used to separate several preservatives, among which imidurea, and skin whiteners in a cosmetic cream. The experimental conditions consisted of a Hibar® RT LiChrosorb® (250 mm×4 mm I.D., 5 μm) 100 CN column (Merck), a mobile phase containing methanol–0.025 *M* phosphate buffer pH 3.0 (40/60 v/v), a 20-μl loop, a detection wavelength of 220 nm and a flow-rate of 1 ml/min. However, at these conditions no retention was observed for imidurea. It can be observed that imidurea also in Ref. [13] did not show a strong retention and was eluting very early in the chromatogram. Several factors were then varied to increase the retention of imidurea. However, neither (i) reduction of the mobile phase solvent strength by decreasing the methanol content (even till 0%), (ii) increase of the pH till pH 7.0, (iii) change of the column (same type, other batch), nor (iv) the use of ion pairing agents (at high and low pH) in the mobile phase (sodium butyl sulfonate, on the first column, and tetrabutylammonium hydrogen sulphate, on the second, in concentrations ranging from 0.01 *M* till 0.05 *M*) were able to cause any retention for imidurea. We will comment on some of the changes introduced. Imidurea has 12 pK_a values of which two are relevant in an aqueous environment, namely 7.35 and 7.97 (pK_a values evaluated with the module ACD/pK_a of the software ACD/ChemSketch (Advanced Chemistry Development Inc., Toronto, Canada) Version 3.60/11 Dec. 1998). They represent the deprotonation of the NH-groups indicated in Fig. 2 to N^--groups. At pH 7.0 a considerable fraction of the molecules is negatively charged and they might interact with the tetrabutylammonium ions, which could affect their retention. The rationale for using butyl sulfonate is less evident, but it was just used to verify whether it would not be able to cause a change in the stationary phase properties so that local positive charges in the imidurea molecule might affect its retention, which, however, was not found to be the case.

Therefore, the idea to assay also imidurea was abandoned and the experimental conditions described by Benassi et al. [9] to analyze free formaldehyde in cosmetics was used as a start to develop an assay for both formaldehyde and ketoconazole. This method used a C_8 column and an acetonitrile–water (1:1, v/v) mobile phase. Since the detection of ketoconazole was not possible at the conditions of Ref. [9] (345 nm), it was performed at 220 nm.

The official EC method for free formaldehyde in the presence of their donor [11] was not taken as a starting point since it was considered to have several disadvantages. The system uses a mobile phase with a rather low pH of 2.1 which will promote a relatively fast deterioration of most reversed-phase silica columns. It also has a mobile phase consisting of 100% buffer which is not supported by most reversed-phases (collapse of C_{18} chains onto the silica surface). Finally it requires a post-column derivatisation which creates a number of practical and technical problems.

The analysis time for ketoconazole was too high under the conditions of Ref. [9] and therefore the water fraction in the mobile phase was replaced by phosphate buffer pH 3.0. This resulted in a faster elution of ketoconazole but also an overlap with the peak of the derivatisation product of formaldehyde. Even though both substances are determined at

different wavelengths and from the injection of differently treated sample solutions we initially preferred to try to separate the substances anyway. The use of the mobile phase described in the Experimental allowed this and was used in the method validation.

The methods to determine ketoconazole and formaldehyde are then validated for the selectivity towards the other excipients in the shampoo, the precision, the linearity range, the bias, the detection limit (if relevant) and the robustness.

3.2. Ketoconazole assay

3.2.1. Selectivity towards excipients

Diluted blank shampoo (250×) was injected and a small peak was observed around the retention time of ketoconazole. It turned out to originate from Comperlan KD. The UV spectrum of this excipient showed a cut-off below 250 nm. Therefore the detection of ketoconazole was performed at 250 nm. At this wavelength the blank shampoo did not show any interfering peaks anymore, while the sensitivity for ketoconazole was only slightly decreased. A chromatogram of 1/250 diluted shampoo detected at 250 nm is shown in Fig. 3.

3.2.2. Linearity

The peak area is linearly proportional to the concentration, up to 0.30 mg/ml. Therefore, in the determination of method repeatability, bias and robustness, the shampoo was diluted 250 times to have an estimated concentration of about 0.08 mg/ml.

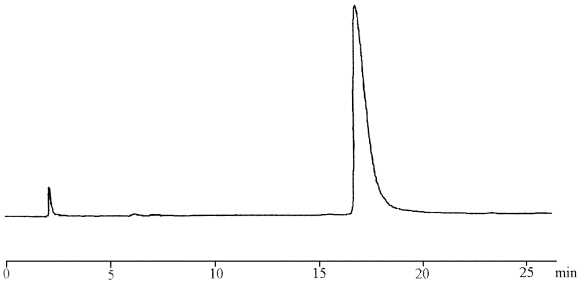

Fig. 3. Chromatogram of 1/250 diluted shampoo. Experimental conditions: see Experimental. Detection wavelength, 250 nm.

3.2.3. Repeatability

The repeatability of injection was determined by the repeated injection of the diluted shampoo ($n=6$). The relative standard deviation (%RSD) on the peak area or on the estimated concentration was found to be 0.50%. The repeatability of the method was determined by analysing six independently diluted samples of one shampoo. The %RSD of the estimated concentrations was 0.37%. The repeatability of the method is comparable to that of injection due to the fact that the ketoconazole assay does not require extensive sample pretreatment.

3.2.4. Bias

The recovery ($n=6$) obtained relative to the theoretical content of 0.02 g/ml was found to be 99.0% and 99.8%, for two independent determinations.

Moreover, the bias of the method was also determined as the percent recovery in three diluted blank shampoo samples spiked with different concentrations of ketoconazole (final concentrations 0.04, 0.08 and 0.12 mg/ml). The samples were analysed in triplicate. The % recovery was calculated as $\%R = C_F/C_A \times 100$ where C_F represents the concentration of analyte measured in the fortified sample and C_A the concentration of analyte added to that sample [17]. The mean recovery rate was found to be 100.4%. From the above results, it could be concluded that no systematic positive or negative bias was found.

3.2.5. Detection limit

The determination of the detection limit is not relevant for this assay.

3.2.6. Robustness

The robustness test was performed on an Alltima (Alltech, Laarne, Belgium) C_8 column (250×4.6 mm I.D., 5 μm). This column was found to have a different selectivity compared to the Nucleosil one on which the method was developed. On the latter, ketoconazole has a longer retention than formaldehyde, while on the Alltima column the opposite is observed. However, given the different sample treatment and detection wavelengths for the ketoconazole and formaldehyde assays, this fact did not cause practical problems. The validation properties on the

Alltima column were found to be similar to those on the Nucleosil column. Linearity was verified up to 0.20 mg/ml, the repeatability of the assay was 1.30%RSD, and the time-different intermediate precision 1.50%.

It turned out that the Nucleosil column, which was used in the method development, has a rather particular selectivity. The method has been tested on two more columns from different manufacturers (Zorbax SB C_8 from Hewlett-Packard and Discovery C_8 from Supelco) and it was found that both had a selectivity similar to that of the Alltima column.

Six factors (parameters) were selected from the analytical procedure to be examined in the robustness test (Table 1). The extreme factor levels were defined symmetrically around the nominal ones. The first six factors in Table 1 are those examined for the ketoconazole assay. The ruggedness test strategy (RTS) program [30,31] was used to define the experimental set-up, to calculate the factor effects from the design results and to interpret them. A Plackett–Burman design for 11 factors requiring 12 experiments was chosen to examine the six selected factors. Five dummy factors were included to complete the design. The solutions injected for each experiment were the calibration standards 0, 0.025, 0.05, 0.10, 0.20 mg/ml of ketoconazole and two dilutions of the shampoo, namely 500 and 200 times.

Table 1
The studied factors and their levels in the robustness tests on the ketoconazole and formaldehyde assays. Factors 1–6 were examined for the ketoconazole assay, factors 1–11 for the formaldehyde assay

Factors	Levels		
	−1	0	1
1 NaH_2PO_4	3.40 g/l	3.45 g/l	3.50 g/l
2 pH	3.8	4.0	4.2
3 ACN	0.43	0.45	0.47
4 Flow	0.9 ml/min	1 ml/min	1.1 ml/min
5 Temp.	25 °C	Room temp.	35 °C
6 Wavelength			
Ketoconazole	249 nm	250 nm	251 nm
Formaldehyde	344 nm	345 nm	346 nm
7 Fraction of HCl	38%	40%	42%
8 pH of buffer	6.6	6.8	7.0
9 Volume DNPH	0.35 ml	0.40 ml	0.45 ml
10 Volume buffer	0.35 ml	0.40 ml	0.45 ml
11 Volume NaOH	0.65 ml	0.70 ml	0.75 ml

NaH_2PO_4, concentration of NaH_2PO_4 in aqueous part of the mobile phase; pH, pH of the aqueous part of the mobile phase; ACN, fraction of acetonitrile in mobile phase; flow, flow-rate of mobile phase; temperature, column temperature; wavelength, detection wavelength; fraction of HCl, fraction of HCl in mixture HCl:H_2O to prepare 2,4-DNPH solution; pH of buffer, pH of buffer used in derivatisation reaction; volume DNPH, volume of 2,4-DNPH solution used in derivatisation reaction; volume buffer, volume of buffer solution in derivatisation reaction; volume NaOH, volume of NaOH added to this reaction.

The following responses were determined for each experiment: the content of ketoconazole in the shampoo calculated from peak area, the capacity factor (k') and the tailing factor (Asf) of the ketoconazole peak. The estimated (E_X) and normalised ($\%E_X$) effects of the factors, and their significance (SF) on the different responses of the ketoconazole assay are shown in Table 2.

The assay can be considered robust because none of the studied factors has a significant effect on the determination of the content of ketoconazole in the shampoo. System suitability test (SST) limits for a number of responses (e.g. k' and Asf) are established as a step following the robustness test ''to ensure that the validity of the analytical procedure is maintained whenever used'' [19]. These limits are the most extreme response values for which, from the robustness test evidence, was found that they still allow a correct quantitative determination under conditions similar to those at which the method validation is conducted (nominal conditions) and on the used column. The use of the results of the worst-case situations, determined from a robustness test, to define SST-limits was proposed earlier [32]. To select the worst-case conditions, the non-significant factors are kept at nominal level while the significant ones are set at the levels which cause the worst result for the considered response. The SST limit for a response can be predicted as the value Y estimated at worst-case conditions:

$$Y = b_0 + \frac{E_{F_1}}{2}F_1 + \frac{E_{F_2}}{2}F_2 + \cdots + \frac{E_{F_k}}{2}F_k \quad (1)$$

with b_0 the average design result, E_{F_i} the effect of the factor considered and F_i the level (-1 or $+1$) causing the worst result. For non-significant factors, the F_i level is set at zero.

Apart from this prediction, the SST limits can also be experimentally determined from replicate experi-

Table 2
Estimated (E_x) and normalised factor effects (%E_x), critical effects ($E_{critical}$, %E_{crit}) and significance (SF) of the factors on the responses measured for the ketoconazole assay

Factors	[C] (area)			Asf			k'		
	E_x (g%)	%E_x	SF	E_x	%E_x	SF	E_x	%E_x	SF
NaH_2PO_4	−0.0025	−0.13	–	−0.010	−0.92	–	−0.440	−14.11	**
pH	0.0072	0.39	–	−0.007	−0.61	–	1.062	34.04	**
ACN	0.0095	0.51	–	−0.208	−18.36	**	−0.467	−14.96	**
Flow	−0.0085	−0.46	–	−0.025	−2.30	–	−0.040	−1.27	–
Temp.	−0.0058	−0.31	–	−0.045	−4.13	–	0.046	1.46	–
Wavelength	−0.0055	−0.30	–	−0.040	−3.67	–	0.080	2.58	–
d_1	0.0035	0.19	–	0.023	2.14	–	−0.120	−3.84	–
d_2	−0.0015	−0.08	–	−0.013	−1.22	–	0.020	0.64	–
d_3	−0.0008	−0.05	–	−0.018	−1.68	–	0.160	5.12	–
d_4	0.0132	0.71	–	0.092	8.41	–	0.031	0.99	–
d_5	−0.0078	0.42	–	0.033	3.06	–	−0.107	−3.42	–
Significance level	$E_{critical}$	%E_{crit}		$E_{critical}$	%E_{crit}		$E_{critical}$	%E_{crit}	
5%	0.0182	0.98		0.118	10.85		0.264	8.45	
1%	0.0285	1.54		0.185	17.01		0.413	13.25	

d_i, dummy factor; –, non-significant effect; *significant at 5% level; **significant at 1% level.

ments at the worst-case conditions. The SST-limits are then defined as the lower or upper limit (depending on what is the worst result for a response) from the one-sided 95% confidence interval around the worst-case mean,

$$\left[\bar{Y}_{\text{Worst-case}} - t_{\alpha,m-1} \cdot \frac{s}{\sqrt{m}}, \infty \right]$$

or

$$\left[0, \bar{Y}_{\text{Worst-case}} + t_{\alpha,m-1} \cdot \frac{s}{\sqrt{m}} \right]$$

with $\bar{Y}_{\text{Worst-case}}$ the average response at the worst-case conditions, m the number of replicates, s the standard deviation of the replicates and $t_{\alpha,m-1}$ the tabulated t-value with $m-1$ degrees of freedom at significance level α. The worst-case experiments were carried out in three independent replicates.

The obtained SST limits from both approaches are summarised in Table 3 and were found comparable.

In summary, the assay for ketoconazole has been validated. The method was found selective relative to the excipients. The linearity, precision and bias were acceptable in the expected concentration range. Furthermore, the robustness test did not indicate any factors to affect the assay.

3.3. Formaldehyde

3.3.1. Selectivity towards excipients

The chromatogram of a 1/100 times diluted shampoo after derivatisation is shown in Fig. 4. Injection of 100 times diluted derivatised blank shampoo and detection at 345 nm did not show any interfering excipient peak.

3.3.2. Linearity

The linearity of formaldehyde calibration curves was examined in different ranges. Linearity was observed in three ranges: 2×10^{-5}–1×10^{-4}%, 1×10^{-4}–1×10^{-3}%, and 1×10^{-3}–5×10^{-3}%. It can also be remarked that the measurement of calibration

Table 3
The SST limits for capacity and tailing factors in the ketoconazole assay

Nominal results			
Asf	k'		
1.08	3.30		
SST limits			
From worst-case results		From theoretical model	
Asf	k'	Asf	k'
1.12	2.68	1.19	2.57

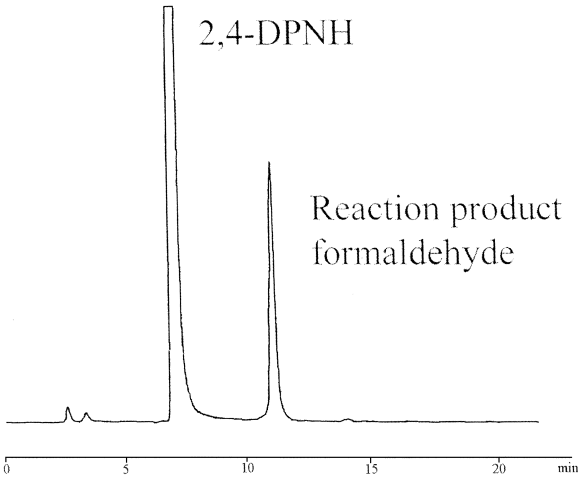

Fig. 4. Chromatogram of 1/100 diluted shampoo after derivatisation with 2,4-dinitrophenylhydrazine. Experimental conditions: see Experimental. Detection wavelength, 345 nm.

curves, especially those in low concentration ranges, should be accompanied by the measurement of a blank. A small formaldehyde peak can namely be observed in blank solutions. This originates from the fact that formaldehyde is a major source of indoor air pollution in North America, Asia and Europe [35]. The World Health Organisation, the American Society of Heating, Refrigerating and Air Conditioning Engineers, the American Lung Association, and many national governments have set a maximum allowable indoor exposure level to formaldehyde at 0.1 ppm [35]. This concentration is in an aqueous solution equivalent to 1×10^{-5}%. Formaldehyde is normally present in low levels, usually less than 0.03 ppm, both in outdoor and indoor air [35].

3.3.3. Repeatability and assay in shampoo

The repeatability of the assay was determined at three concentration levels of formaldehyde, namely in 500, 100 and 10 times diluted shampoo, by analysing six independently prepared samples at each level. The %RSD of the concentrations calculated from peak area was found to be 2.81% in 500 times diluted shampoo, 0.67% in 100 times, and 1.46% in 10 times diluted shampoo. Determination of the repeatability in the 100 times diluted shampoo on a different day gave a %RSD of 1.21%.

The content of formaldehyde in all these samples was also estimated, relative to an appropriate calibration line for a given shampoo dilution. The estimated formaldehyde concentration ($n=6$) in the shampoo estimated from the 500 times diluted shampoo was 0.038 g/100 ml (0.038%), 0.039% was found twice from the 100 times diluted shampoos and 0.034% for the 10 times diluted samples. Besides, in other dilutions of the sample ($n=1$) formaldehyde concentrations were estimated as 0.038% (1000 and 250 times dilutions), 0.037% (250× diluted), 0.039% (50× diluted) and 0.036% (25× dilution).

To evaluate occasional matrix effects a standard addition calibration line (ΔC ranging from 3×10^{-4}–15×10^{-4}%, five standards) was prepared in 100 times diluted shampoo. The concentration of formaldehyde estimated in the shampoo was 0.035%, which is similar to the concentrations found using the external calibration lines.

3.3.4. Bias

To determine the bias of the method 4×10^{-4}% formaldehyde was added to 100 times diluted shampoo samples ($n=3$). The diluted shampoo and the spiked samples were then analysed. The mean recovery was found to be 101.8%. Also 3×10^{-4}% and 6×10^{-4}% spikes were made ($n=1$). The recoveries found then were 105.0% and 100.4%, respectively.

3.3.5. Detection limit

Formaldehyde concentrations below 2×10^{-5}% still were derivatised but the resulting peak was not proportional to the concentration anymore. The detection limit is at least 4×10^{-6}% since this concentration still causes an important increase in the peak compared to the one observed in a blank.

3.3.6. Robustness

The robustness test was again performed on the Alltima C_8 column. On this latter column linearity was observed in the same ranges as on the Nucleosil one. Repeatability of the assay was 2.28%RSD for 500 times diluted shampoo and 1.98% for 100 times diluted, while the time-different intermediate precision was 3.40% and 3.06%, respectively. Eleven factors were selected, of which the first six were the same as those in the robustness test of the

ketoconazole assay (Table 1). The other five were related to the derivatisation reaction of formaldehyde. These 11 factors were examined in a Plackett–Burman design for 15 factors with 16 experiments, which required four dummy factors to complete the design.

Due to the different volumes of 2,4-DNPH reagent, of phosphate buffer and of NaOH added to the reaction mixture, the final volume was not the same for each experiment. Therefore, for each derivatisation reaction, the total volume was adjusted with water to 2.65 ml, which is the highest volume required by the design.

In each experiment, the injected solutions were standards to create a calibration line in the interval 2.5×10^{-5}–10×10^{-4}% formaldehyde and a shampoo sample at two dilutions, 500 and 100 times. The responses measured or calculated were the capacity and tailing factor of the formaldehyde peak, the resolution between the 2,4-DNPH reagent peak and the one of formaldehyde, and the content of formaldehyde in the shampoo estimated both from peak area and height.

The factor effects on the different responses are shown in Table 4. Regardless of whether peak area or height was used to calculate the content of formaldehyde in the shampoo, the responses were not significantly influenced by any of the examined factors at $\alpha=5\%$. Therefore, the assay of formaldehyde can be regarded as robust.

Several factors were found to have significant effects on the responses capacity factor, tailing factor and resolution. It can be seen that neither the factors dealing with the derivatisation reaction, nor the factor ''detection wavelength'' nor the four dummies are indicated as significant for any of the responses. The SST limits for these three responses were then determined from experiments at the worst-case conditions and from the theoretical model (Eq. (1)). The results are shown in Table 5.

In summary, it can be concluded that this method is robust with respect to the examined factors since the content estimation of formaldehyde was not affected by the introduced factor changes. Furthermore, the other validation characteristics can be regarded as acceptable and the separation between

Table 4
Estimated (E_X) and normalised effects ($\%E_X$), critical effects ($E_{critical}$, $\%E_{crit}$) and significance (SF) of the factors on the responses measured for the formaldehyde assay

Factors	[C] (area)			[C] (height)			Asf			k'			Rs		
	E_X (10^{-4}%)	$\%E_X$	SF	E_X (10^{-4}%)	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
NaH_2PO_4	0.087	2.54	–	0.012	0.34	–	−0.005	−0.55	–	0.240	4.50	–	0.624	3.38	–
pH	0.054	1.59	–	0.110	3.21	–	0.063	6.48	**	−0.293	−5.49	–	−0.209	−1.13	–
ACN	0.130	3.81	–	0.016	0.46	–	0.079	8.18	**	−0.974	−18.25	*	−1.929	−10.45	*
Flow rate	0.056	1.64	–	−0.019	−0.56	–	0.006	0.66	–	−0.127	−2.38	–	−0.269	−1.46	–
Temperature	−0.118	−3.46	–	−0.098	−2.86	–	−0.066	−6.77	**	−0.744	−13.94	*	−1.069	−5.79	–
Wavelength	−0.085	−2.48	–	−0.029	−0.85	–	−0.018	−1.89	–	0.101	1.90	–	0.349	1.89	–
Fraction of HCl	−0.004	−0.10	–	0.030	0.86	–	0.023	2.41	–	0.110	2.06	–	0.356	1.93	–
pH of buffer	−0.053	−1.54	–	−0.011	−0.33	–	0.017	1.77	–	−0.284	−5.32	–	−0.416	−2.26	–
Volume DNPH	0.094	2.73	–	0.065	1.90	–	0.003	0.35	–	0.160	3.00	–	0.086	0.47	–
Volume buffer	−0.018	−0.51	–	−0.051	−1.47	–	−0.022	−2.23	–	0.227	4.26	–	0.536	2.91	–
Volume NaOH	−0.006	−0.19	–	0.033	0.97	–	−0.005	−0.55	–	0.038	0.71	–	0.384	2.08	–
d_1	0.037	1.09	–	−0.023	−0.67	–	0.009	0.89	–	−0.189	−3.55	–	−0.466	−2.53	–
d_2	0.017	0.49	–	0.067	1.96	–	0.013	1.33	–	−0.366	−6.86	–	−0.834	−4.52	–
d_3	0.038	1.12	–	0.061	1.76	–	0.012	1.25	–	−0.251	−4.70	–	−0.586	−3.18	–
d_4	0.113	3.31	–	0.040	1.16	–	0.001	0.06	–	−0.051	−0.96	–	−0.311	−1.69	–
Significance level	$E_{critical}$	$\%E_{crit}$		$E_{critical}$	$\%E_{crit}$		$E_{critical}$	$\%E_{crit}$		$E_{critical}$	$\%E_{crit}$		$E_{critical}$	$\%E_{crit}$	
5%	0.175	5.12		0.141	4.11		0.0273	2.82		0.673	12.62		1.615	8.75	
1%	0.291	8.50		0.234	6.81		0.0453	4.67		1.117	20.93		2.678	14.51	

d_i, dummy factor; –, non significant effect; *significant at 5% level; **significant at 1% level.

Table 5
The SST limits for the resolution, the capacity and tailing factors in the formaldehyde assay

Nominal results					
Asf	k'	Rs			
1.04	5.26	18.8			
SST limits					
From worst-case results			From theoretical model		
Asf	k'	Rs	Asf	k'	Rs
1.11	4.36	17.0	1.11	4.02	16.8

the reagent and formaldehyde peaks showed a good resolution.

4. Conclusions

Ketoconazole and formaldehyde could be determined in the same chromatographic system. Acceptable method validation results were obtained. Different sample pretreatment and detection conditions are needed for each of the substances. This means that the determination of ketoconazole is not affected by formaldehyde and vice versa. It turned out that the latter was an advantage since considerably different selectivities between formaldehyde and ketoconazole can be found on different columns.

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