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Short communication

Validation of an analytical method for quality control of residual solvents (*n*-hexane and acetone) in D-002: New active ingredient from beeswax

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Abstract

D-002 is a new natural product consisting of a mixture of aliphatic fatty alcohols, which shows antioxidant and anti-ulcer effects in experimental models. A new validated methodology for determining simultaneously residual *n*-hexane and acetone in D-002 using the headspace gas chromatography (HS/GC) is described. The very poor solubility of D-002 in most solvents did necessary sample preparations in solid state. Limit test conditions allowed a detection of residual *n*-hexane and acetone more sensitively than that recommended for such purposes in the general method of the European Pharmacopoeia. Validation assays, applied to both D-002 residual solvents, proved: suitable sensitivity; very high linearity (correlation coefficients $\geq$0.999, R.S.D. of slopes $\leq$0.8% and R.S.D. of response factors $\leq$5% and no biases) and accuracy (average recoveries between 94.7 and 100.1%); and precision was $\leq$2.1%. The method was found suitable for quality control and stability studies of this new product.

Keywords: D-002; Residual solvents; HS/GC; Limit test; Validation

1. Introduction

Today more than ever the medical regulating entities, to guarantee the security to the consumers, are paying special attention to the topic of the residual solvents present in the active ingredients and pharmaceutical products. For this reason, it is necessary to have suitable methods for the residual solvent controls for those new products that are developed. These methods must fulfill the current international regulations, and this way such products do not represent a risk for the health of the patients.

In December 1997, the International Conference on Harmonization (ICH) published their guidance for the Industrial Q3C Impurities, which was addressed to the topic of residual solvents in pharmaceutical products [1]. The ICH Q3C guidelines classify the residual solvents into classes according to highest to lowest human risk, and define its corresponding acceptance limits also. All the substances and products should be examined as for its probable content of residual solvent, what will be subordinated to those that are used during their respective production.

With regard to revising the test 467 related with the organic volatile solvents, the United States Pharmacopoeia adopted finally the rules of the Q3C on 1 July 2007. On the contrary, from the year 2000, European Pharmacopoeia had already adopted the same principles contained in the Q3C for active ingredients, excipients and pharmaceutical products [2].

D-002 is a new natural active ingredient consisting of a mixture of aliphatic saturated fatty alcohols [3], with antioxidant [4–6] and anti-ulcer effects [7–9], isolated and purified from beeswax. In the process of obtaining the D-002, both *n*-hexane and acetone are used, which are class 2 and 3, respectively. It becomes necessary to have a validated analytic methodology that allows having a criterion of quality with regard to the contents of residual solvents that even after drying stages they could stay. So, by means of this methodology, one will be able to detect if some batch presents bigger residual contents than the permissible limits of the ICH guidance. When the *n*-hexane and acetone are combined in a pharmaceutical product as residual solvents, its maximum concentration limits are 290 and 5000 ppm, respectively.

Taking into account all the above-mentioned, an analytic method by which is controlled simultaneously the residual *n*-hexane and acetone contents in D-002 active ingredient was

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subject to validation. The main validating results are presented in the current paper.

2. Experimental

2.1. Materials

D-002 (batch 030020206) randomly selected and recently produced in Group #3 (CNIC, Cuba), *n*-hexane (99.9%, Riedel-de Haen, Germany), acetone (99.8%, Riedel-de Haen, Germany), carbon bisulphite (99.9%, Merck, Darmstadt, Germany) and anhydrous sodium sulphate (99%, Merck, Darmstadt, Germany).

The following solutions were prepared—*n*-hexane reference solution (HRS): an aliquot of 0.1 ml of *n*-hexane (density, 660 g l^{-1} at 20 °C) was added to a 50 ml volumetric flask. The volume was completed with carbon bisulphite, shaking gently to homogenize; acetone reference solution (ARS): 1 ml of acetone (density, 790 g l^{-1} at 20 °C) was added to a 25 ml volumetric flask and the volume was completed with carbon bisulphite, shaking gently to homogenize; working standard solution of *n*-hexane (WSSH): 20 μl of *n*-hexane was added to a 100 ml volumetric flask. The volume was completed with carbon disulphide, shaking gently to homogenize. An aliquot of 8 ml of this solution was transferred to a 50 ml volumetric flask. The volume was completed with carbon disulphide, shaking gently to homogenize; working standard solution of acetone (WSSA): 50 μl of acetone was added to a 50 ml volumetric flask. The volume was completed with carbon disulphide, shaking gently to homogenize. These solutions were found to be stable for at least 1 month when stored at +8 °C.

2.2. Chromatographic conditions

Headspace equipment K-MAS 5 (Konik, Barcelona, Spain) coupled to a gas chromatograph model HRGC 4000B (Konik, Barcelona, Spain) with flame ionisation detector. The operational parameters of the headspace auto-sampler were as follows: desorption temperature (80 °C), desorption time (10 min), temperature at the transference line (120 °C), temperature of the commutation valve (120 °C), pre-injection time (4 min), injection time (22 s.), pressure (20 psi), flow of carrier gas (helium) was 4 ml min^{-1}. Whereas the chromatographic conditions were as follows: a BP-1 wide bore column (30 m, 0.53 mm i.d., 3.0 μm D_f; SGE, Texas, USA) was used from 30 °C (1 min isothermal) to 200 °C (4 °C min^{-1}). Injector and detector were set at 200 °C. To form the flame, hydrogen and airflow were 38 and 280 ml min^{-1}, respectively.

2.3. Assessment of the solvent chromatographic peaks

The presence of the *n*-hexane and acetone chromatographic peaks was assessed through the 95% confidence interval (CI) of its retention times (t_r). For this, an *n*-hexane reference (HR) was prepared by adding 0.5 μl of *n*-hexane to a 10 ml vial, which was sealed and shaken gently for 1 min to homogenize. An acetone reference (AR) was also prepared by the same requirements.

A blank sample was prepared by injecting 2 μl of carbon bisulphite into the solid sample (1 g of anhydrous sodium sulphate) placed into a 10 ml vial hermetically sealed, shaking gently for 1 min to homogenize. Blank sample was always assessed through the HS/GC system to discard any contamination. No signal or peak should be expected.

The HR and AR were independently assessed five times through the HS/GC system for determining the CI of the t_r for *n*-hexane and acetone, respectively, which were calculated as follows:

$$\mathrm{CI} = \frac{t_r \pm t \times \mathrm{S.D.}}{n^{1/2}}$$

where t is the value of the Student's t distribution for $n-1$ freedom degrees for a $P = 0.05$, n = number of analyses.

Then, different samples were prepared weighing 1 ± 0.001 g of D-002 into different 10 ml vials that were hermetically sealed, which were analysed in the HS/GC system. Thereafter, chromatograms of the HRs and ARs were compared with those of D-002 samples to detect the presence in the latter of chromatographic responses that appear within the t_r intervals of residual solvents. Chromatographic responses of both residual *n*-hexane and acetone should be at least 10 times greater than the background noise.

2.4. Linearity study

Two linearity studies were conducted using five concentration levels, each point made by triplicate. The regression lines were obtained from the responses (peak areas) of residual solvent (y) *versus* their concentrations in ppm (x). In this sense, the samples prepared for each point of the calibration curves were assessed as follows: 1 ± 0.001 g solid samples of anhydrous sodium sulphate were accurately weighted in 10 ml vials, which were sealed hermetically. Then, five *n*-hexane concentrations (0.02, 0.04, 0.06, 0.08 and 0.10 ppm) and five acetone concentrations (0.79, 2.37, 3.95, 7.11 and 7.9 ppm) were assessed in combined way. For which 1, 2, 3, 4 and 5 μl aliquots of the WSSH, and 1, 3, 5, 9 and 10 μl aliquots of the WSSA, were injected into the solid samples in paired form (from lowest to highest concentration). After that, each sample was shaken gently to homogenize.

Linearity and proportionality tests established a $P = 0.05$ and the following acceptance criteria: correlation coefficient (r) ≥ 0.99; relative standard deviation of response factor (R.S.D.$_f$) $\leq 5\%$, where response factor is defined as y/x; and relative standard deviation of slope (R.S.D.$_b$) $\leq 2\%$, with

$$\mathrm{R.S.D.}_b\,(\%) = \frac{\mathrm{S.D.}_b}{b} \times 100$$

where b is the slope, and S.D.$_b$ is the standard deviation of the slope.

To prove that no bias was present, the zero value should be included in the CI of the intercept (a), which was calculated as follows:

$$\mathrm{CI} = a \pm t \times \mathrm{S.D.}_a$$

where S.D.$_a$ is the standard deviation of the intercept, and t is the value of the Student's t distribution for $n-2$ freedom degrees for a $P=0.05$.

The results were not valid unless the R.S.D. of the solvent peak areas was lower than 15% in all the evaluated concentrations.

2.5. Concentration of residual solvents in D-002

The concentration of *n*-hexane and acetone in D-002 samples was determined. For that, five independent 1 ± 0.001 g samples of D-002 were placed in 10 ml vials and sealed hermetically. Then, 2 μl aliquots of carbon disulphide was injected through the seal into the solid samples, shaken gently for 1 min to homogenize, to reproduce the conditions of the linearity assessment.

The *n*-hexane and acetone concentrations (ppm) in the D-002 were determined interpolating the average of *n*-hexane and acetone responses of the batch in the calibration curves of the *n*-hexane and acetone linearity tests, respectively. The results were not valid unless the R.S.D. of the peak areas was lower than 15% by each solvent.

2.6. Quality control method of residual solvents in D-002 (limit test)

2.6.1. Sample preparations

D-002 test sample (DTS): it was accurately weighted a sample of the D-002 batch (1.00 ± 0.001 g) in a 10 ml vial, which was sealed hermetically. Then, 2 μl of carbon bisulphite was injected inside the D-002 sample previously weighed into the vial. After that, it was shaken gently manually for 1 min to homogenize. The preparations were in triplicate.

Solvent reference sample (SRS): it was accurately weighted a sample of the D-002 batch (1.00 ± 0.001 g) in a 10 ml vial, which was sealed hermetically. Then, 1 μl of HRS and the same quantity of ARS were injected inside the D-002 sample previously weighed into the vial. After that, it was shaken gently manually for 1 min to homogenize. The preparations were in triplicate.

2.6.2. Assessment of residual solvents

After determining the t_r values of *n*-hexane and acetone, and corroborating the cleaning of the HS/GC system, the DTSs and SRSs were assessed. After every run, blank sample was analysed, and no signal or peak should be expected in the t_r values of the residual solvents.

With regard to obtaining the chromatographic responses, if the mean values of solvent peak areas in the DTSs were lower than the half of those obtained in the SRSs, the residual *n*-hexane and acetone in the D-002 batch should be lower than 1.32 and 31.5 ppm, respectively. The R.S.D. of the differences in the areas among the residual solvent peaks obtained in the three replicate paired injections of SRS and DTS should be $\leq$15%.

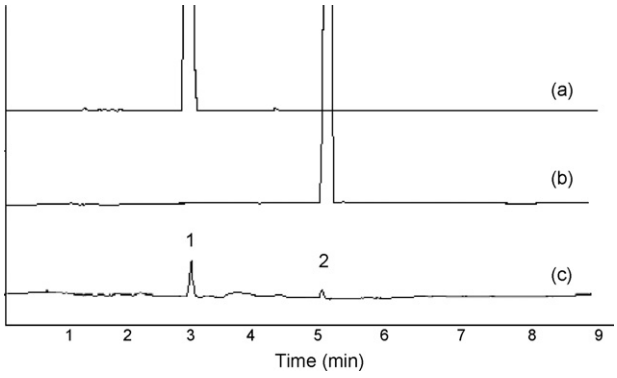

Fig. 1. HS/GC profiles of AR (a), HR (b), and D-002 (c) analysed under the method conditions. Peaks 1 and 2, residual acetone and *n*-hexane, respectively.

2.7. Accuracy study

Accuracy was assessed for both residual *n*-hexane and acetone by a recovery study.

Spiked samples were prepared, weighted accurately (1.00 ± 0.001 g) of D-002 batch in 10 ml vials, which were sealed hermetically. Then, volumes of 0.1, 0.5 and 1 μl of HRS and the same quantities of ARS were injected in paired way (from lowest to highest concentration) inside the D-002 samples previously weighed into the vials. After that, it was shaken gently manually for 1 min to homogenize. DTSs were used as blank samples. The preparations were in triplicate.

The differences in the responses among the residual solvents obtained in the three replicate paired injections of spiked samples and DTSs were interpolated as per the solvent in the calibration curves of the linearity tests. Mean recoveries were checked to 100% with the Student's t-test for $P=0.05$. The experimental t (t_{exp}) values were calculated as follows:

$$t\,\mathrm{exp} = \frac{|100 - \mathrm{recovery}|\sqrt{n}}{\mathrm{R.S.D.}}.$$

3. Results and discussion

3.1. Assessment of the solvent chromatographic peaks

Fig. 1 shows the HS/GC profiles of the HR, AR and D-002 sample obtained under method conditions. As can be seen, chromatographic responses within the CI of the t_r of the solvents in the chromatograms of D-002 samples were detected without interferences that masked such responses.

The response of *n*-hexane in D-002 samples was about 35 times greater than the average background noise, and therefore, more than three times greater than the response equivalent to the quantification limit, defined as the concentration of the solvent that produces a response 10 times the average noise background [10]. Likewise, the acetone response was about 410 times greater than the average background noise. Then, the method conditions guaranteed to be able to make determinations of residual *n*-hexane and acetone at lower concentrations than the real ones.

Table 1
Chromatographic responses vs. solvent concentrations

n-Hexane assessment			Acetone assessment		
Concentration (ppm)	Mean ± S.D. (μV s)	R.S.D. (%)	Concentration (ppm)	Mean ± S.D. (μV s)	R.S.D. (%)
0.02	848 ± 85	10.0	0.79	13249 ± 964	7.3
0.04	1637 ± 62	3.8	2.37	41390 ± 1058	2.6
0.06	2600 ± 77	3.0	3.95	69882 ± 4929	7.0
0.08	3423 ± 66	1.9	7.11	119859 ± 6537	5.4
0.10	4186 ± 36	0.9	7.90	135168 ± 1940	1.4

3.2. Linearity study

Table 1 shows the concentrations of *n*-hexane and acetone, at each point of the calibration curves and their respective means of the chromatographic responses. The R.S.D. values of the responses were lower than 15% in both solvents.

The regression lines obtained for *n*-hexane and acetone are shown in Table 2. For both solvents, the values of *r*, R.S.D.$_f$ and R.S.D.$_b$ fulfilled with their acceptance criterion ($\geq$0.99; $\leq$5.0% and $\leq$2.0%, respectively). Also, the CI of the intercepts included the zero value, therefore, no bias occurred. According to these results, the method was able to obtain linear proportional responses at the very low concentrations of *n*-hexane and acetone assessed.

3.3. Concentration of residual solvents in D-002

In pharmacopoeias, the general procedures described for identification and control of residual solvents are not based on a determination of the accurate content of these in active substances, but they are intended on the basis of a limit test, in which without reaching such determination, it is proved that the content of the residual solvent is lower than their maximum ICH limit. Nevertheless, we included in the current paper the analysis of the exact concentrations of residual *n*-hexane and acetone in D-002 samples.

In this sense, the average chromatographic response of *n*-hexane in the D-002 samples was 1139 μV s with R.S.D. of 8.7%. The latter R.S.D. was lower than the permissible criterion ($\leq$15%). With regard to interpolating the average *n*-hexane response in the *n*-hexane curve (linearity test), the concentration of residual *n*-hexane in the batch was of 0.03 ± 0.005 ppm.

On the other hand, the average chromatographic response of acetone in the samples of the batch was 14011 μV s with R.S.D. of 4.3%. The same as the previous *n*-hexane case, R.S.D. value in residual acetone determination was lower than the permissible criterion. When interpolating this average chromatographic response in acetone curve, the batch had contents of residual acetone of 0.8 ± 0.03 ppm.

The results proved that the D-002 batch had residual *n*-hexane and acetone contents much lower than the upper acceptance limits of ICH guideline (290 and 5000 ppm, respectively).

3.4. Quality control of residual solvents in D-002

The limit test was developed taking into account the general method described in the European Pharmacopoeia for such purposes [2]. However, it was necessary to make some adjustments to this method due to the very poor solubility of D-002 in most solvents. That is why, the procedure did not involve a dissolution of D-002 in water R or *N*,*N*-dimethylformamide R as the pharmacopoeia describes but the samples were analysed in solid state. In the procedure applied, moreover, the extra *n*-hexane and acetone concentrations in the SRS were as low as of 1.32 and 31.5 ppm, respectively, lower than the concentrations of these solvents (2.42 and 41.7 ppm, respectively) that should contain the SRS as per the pharmacopoeia method. Then, the developed method demanded a detection of residual *n*-hexane and acetone more sensitively than those recommended in the general method of the European Pharmacopoeia.

The average chromatographic responses of *n*-hexane and acetone in the DTSs were lower than the half of their average responses in the SRSs (Table 3), therefore, the residual *n*-hexane and acetone concentrations were lower than 1.32 and 31.5 ppm, respectively. These results were expected, since the residual *n*-hexane and acetone concentrations in the batch, calculated by interpolation in the calibration curves, were 0.03 and 0.8 ppm, respectively, values much lower than the maximum limits of the ICH guideline.

The R.S.D. values assessed through the differences of the solvent responses among DTSs and SRSs were lower than 2.1%, which supports that the method was precise enough.

Table 2
Linearity of HS/GC determinations of *n*-hexane and acetone

Solvent	$y = (b \pm t \times \text{S.D.}_b)x \pm (a \pm t \times \text{S.D.}_a)$	*r*	R.S.D$_f$ (%)	R.S.D$_b$ (%)
n-Hexane	$y = (42186 \pm 457)x + (13.5 \pm 30.3)$	0.9995	4.8	0.5
Acetone	$y = (17057 \pm 305)x + (365 \pm 1584)$	0.9990	5.0	0.8

a, intercept; *b*, slope; *r*, correlation coefficient.

Table 3
Results obtained after applying the *n*-hexane and acetone limit test with the D-002 batch

Residual solvent	Sample	t_r (min)	Response (μV s)	Average response ± S.D. (μV s)	R.S.D.[a] (%)
Acetone	DTS-1	3.117	13066	13218 ± 546	1.2
	DTS-2	3.117	12765		
	DTS-3	3.100	13824		
	SRS-1	3.067	549673	551526 ± 5878	
	SRS-2	3.133	558108		
	SRS-3	3.100	546799		
n-Hexane	DTS-1	5.283	1024	1135 ± 97	2.1
	DTS-2	5.283	1175		
	DTS-3	5.217	1206		
	SRS-1	5.183	52660	53786 ± 1187	
	SRS-2	5.267	53673		
	SRS-3	5.200	55026		

[a]R.S.D. value assessed through the differences of the residual solvent responses of DTS and SRS.

Table 4
Accuracy of HS/GC determinations of residual *n*-hexane and acetone in D-002

Residual solvent	Amount added (μg)	Amount found (μg)			Mean recovery ± S.D. (%)	t_{exp}[a]
		1	2	3		
n-Hexane	0.13	0.10	0.12	0.14	92.3 ± 15.4	0.799
	0.66	0.75	0.60	0.80	108.6 ± 15.8	1.027
	1.32	1.22	1.24	1.28	94.4 ± 2.34	3.911
Acetone	3.15	3.10	3.12	3.25	100.2 ± 2.62	0.133
	15.75	15.70	15.73	15.85	100.1 ± 0.47	0.368
	31.5	31.44	31.95	31.22	100.1 ± 1.19	0.175

[a] Experimental *t*; tabulated $t = 4.303$ (0.05;2).

3.5. Accuracy study

The mean recoveries for both *n*-hexane and acetone were between 92.3–108.6% and 100.1–100.2%, respectively. The t_{exp} values were lower than tabulated *t* for $P = 0.05$ (Table 4), so the recoveries and 100% values were not significantly different. Then, despite the very low concentrations of the extra *n*-hexane and acetone added in the spiked samples, the method was able to make accurate determinations of them.

4. Conclusions

The analytical method proposed for the quality control of D-002 active ingredient, on account of the residual *n*-hexane and acetone contents, complied with the requirements to be considered validated. That is, it demonstrated to be sensitive, linear, accurate and precise.

A recent D-002 batch randomly selected was analysed under validated method conditions, being proved that the contents of residual *n*-hexane and acetone are much lower than their maximum ICH limits, and therefore their presence is not by far a risk to the health of the consumer.

References

[1] Proceedings of International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Tripartite Harmonised guideline Q3C Impurities: Residual Solvents, 1997.
[2] European Pharmacopoeia, Supplement, third ed., Council of Europe, 67075 Strasbourg Cedex, France, 2000.
[3] V. Gonzales, R. Sierra, J. Magraner, E. Rodríguez-Leyes, Rev CENIC. Ciencias Quím. 37 (2006) 35–41.
[4] R. Menéndez, R. Mas, J. Illinait, J. Perez, A.M. Amor, R.M. Gonzalez, J. Med. Food 4 (2001) 71–77.
[5] S. Mendoza, M. Noa, Y. Pérez, R. Mas, J. Med. Food 10 (2007) 379–383.
[6] R. Menéndez, R. Mas, A.M. Amor, Y. Perez, R.M. González, J.C. Fernández, S. Jiménez, Arch. Med. Res. 32 (2001) 436–441.
[7] J. Illinait, H. Terry, R. Mas, L. Fernandez, D. Carbajal, J. Med. Food 8 (2005) 63–78.
[8] V. Molina, D. Carbajal, M.L. Arruzazabala, R. Más, J. Med. Food 8 (2005) 59–62.
[9] D. Carbajal, V. Molina, L. Arruzazabala, R. Mas, Rev CENIC. Ciencias Quím. 34 (2005) 15–21.
[10] Validation of Analytical Procedures: Methodology, Harmonized Tripartite, Guideline, Topic Q2B UK, Step 4, 1996.