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TESTING THE IN VITRO EQUIVALENCE OF TWO ACETYLCYSTEINE BASED PRODUCTS

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ABSTRACT. The main objective of this study was to compare two acetylcysteine based products with identical quantitative and qualitative composition (acetylcysteine 2g, sucrose and vanillin) and identical pharmaceutical form (oral powder). For the equivalence of the two items we chose the dilution profiles method of determined through HPLC. The data obtained was statistically analyzed and for interpretation, the f2 similarity factor was calculated. We resorted to using this test procedure in vitro because through HPLC, we could not determine the plasmatic concentrations of the 10 horses tested in vivo, in the initial implementation of a standardized protocol. Average percentages of the samples of tested and reference products, dissolved for test showed more than 85% after 5 minutes in each of the 3 different pH dissolution media, indicating similar dilution profiles, indicating there was no need for calculating the f2 similarity factor. Based on the obtained in vitro dissolution profiles, identical quantitative and qualitative composition (2 g acetylcysteine in 6 g powder) and the identical pharmaceutical formulation (oral powder) in vivo testing of the two investigated products is not necessary.

Keywords: bioequivalence, *in vitro*, acetylcystein, horse, f_2 similarity factor

INTRODUCTION

Developments in bioequivalence veterinary products have facilitated the application of these tests in pharmaceutical practice, increasing the safety of the comparison of similar medicinal formulas. For safety evaluation and certification of products introduced in medical therapy and veterinary the use of bioequivalence is recommended in at least 3 cases: when the proposed marketed dosage form differs from the form used in pivotal clinical trials; when substantial changes are made to the manufacturing process of the marketed drug product and when developing a generic formulation of a marketed drug product (Smith, Brian, 2003). According to researchers in the field by bioequivalence are compared pharmacokinetics and pharmacodynamics of the different active substances and active metabolites. Bioequivalence can rely only on in vitro testing for test products identical to the reference, in terms of quantity and quality, thus containing the same active substance. Such tests are sufficient for products whose excipients do not influence absorption of the active substance or the therapeutic effect.

MATERIALS AND METHODS

The main objective of this study was to compare the two acetylcystein based products, identical from a quantitative, qualitative (acetylcystein 2g, sucrose and vanillin) and pharmaceutical form (oral powder) point of view.

For the equivalence of the two items we chose the method of dissolution profiles, based on the use of similarity factor f2. We resorted to using this test procedure in vitro through the HPLC method, because we could not determine acetylcysteine plasma concentrations of the 10 horses tested in vivo, in the initial implementation of the standardized protocol (table 1).

The dissolution profiles were investigated by using a rotor blade, capable of providing 100 rpm. The dissolution was done in 3 media, prepared as described in European Pharmacopoeia, 1000 ml of each buffer solution each, but with different pH: 1.2, 4.6 and 6.8.

From the two products (test and reference) we prepared a total of 12 samples of 6 grams per medium buffer solution. 5 ml samples of solution were then taken at intervals of 5, 10, 20 and 30 minutes each time replacing the amount extracted from the dissolution. In calculating the resultant the additional dilution obtained each harvest was taken into account for the average value and standard deviation. Finally, from each sample the amount of acetylcysteine was determined by high performance liquid chromatography (HPLC).

Note that the procedure for dissolution of the products, test and reference, was done in exactly the same conditions, sampling times were identical. To ensure relevance at least 3 sampling times were taken into account. However, calculating the similarity factor does not allow the use of more than an average over 85% of the dissolved substance, for each form. Standard deviation of the mean from each time must be less than 10%, except for the first sampling time, when it can be less than 20%.

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Table 1

Standardized protocol for in vivo testing of an acetylcysteine based product on horses.

Name of active ingredients:	Acetylcysteine 2000 mg (2 g)
Study design	Two period, two sequence, cross-over, block randomized, multiple-dose comparative bioavailability study on healthy horses
Planned sample size	At least 10 horses
Planned number of enrolled subjects	10 horses (5+5)
Main selection criteria	Male and female healthy horses of the same breed, body weight within 400 - 650 kg
Dosage administered/phase	10 mg/kg body weight acetylcysteine in one dosage of TEST formulation or REFERENCE formulation
Route of administration **Duration of treatment**	Oral Two single oral doses (washout 7-14 days)
Primary parameters	AUC0-t and C_{max} of acetylcysteine
Secondary parameters	T_{max}, AUC0-inf
Additional parameters	% extrapolated AUC, thalf, MRT
Safety parameters	Adverse events, clinical and laboratory screening and follow up examinations
Study procedure	Each horse will receive in a random way a single oral dose of TEST or REFERENCE products on two different occasions separated by a wash-out period of 7-14 days. Blood samples will be drawn predose (0.0) and 0.5, 1.0, 1.5, 2, 4, 8, 12, 24, 36, and 48 hours after drug administration.
Analysis of samples	HPLC validated methods, with UV detection technique
Statistical analysis	ANOVA (model: treatments, sequences, subjects, and period of administration) after log transformation for primary pharmacokinetic parameters, with determination of 90% confidence intervals for the intra-individual ratios (test/reference) for primary parameters. Equivalent non-parametric methods for T_{max}.
Acceptance range	AUC0-t, C_{max}: 70-135% log-transformed data

For calculating the similarity factor f2 we used the following formula (Vinod et al., 1998):

$$f_2 = 50 * \log \frac{100}{\sqrt{1 + \frac{\sum_{t=1}^{t=n} \left[\bar{R}(t) - \bar{T}(t) \right]^2}{n}}}$$

n - number of points (sampling times);

$\bar{R}(t)$ - percentage of active substance dissolved in case the reference product (arithmetic mean of 12 samples);

$\bar{T}(t)$ - percentage of active substance dissolved in case the product test (arithmetic mean of 12 samples).

According to the demonstrated and accepted principles based on the value of f_2, a value between 50-100 in dissolution profiles, results in a difference of less than 10% and it can be considered that the two products have similar dissolution profiles. In contrast, if the value of f_2 was below 50, and the difference between dissolution profiles is more than 10%, it can be said that the two products do not have similar dissolution profiles (Vinod et al., 1998).

Finally, if the percentage of dissolved active substance resulted from the average of 12 samples test and reference products, is equal to or greater than 85% after 15 minutes, dissolution profiles can be accepted as similar without further mathematical assessment (Vinod et al., 1998).

RESULTS AND DISCUSSIONS

Regarding the results obtained samples from in vivo testing on healthy horses, it is mentionable only the fact that although we strictly followed the protocol, no plasma levels were detected in all 11 prepared plasma samples at the mentioned intervals and the obtained values were very low therefore irrelevant. These results do not exclude the possibility that acetylcysteine may be inactivated by light, which would mean that the plasma must be prepared in the dark (Hui-chang Bi et al., 2005)

In case of the in vitro testing of the acetylcysteine dissolution of test and reference products in different pH dilution environments we obtained similar values, which are presented in tables 2, 3 and 4 and plotted in figures 1, 2 and 3. As reflected in the data below the values obtained were slightly influenced by time and pH.

Individual data obtained for dilution in 1.2 pH medium revealed an insignificant upward trend in the average values of the percentage of acetycysteine for both products (table 2, fig. 1).

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 20, issue 1, 2010, pp. 75-80

Thus, recorded mean percentage values for the test product were following: 99.82 ± 0.216 at 5 minutes, 100 ± 0.126 at 10 minutes, 100.07 ± 0.080 tat 20 minutes and 100.27 ± 0.123 at 30 minutes. Similar developments were also noted in case of the reference product: 100.02 ± 0.092 at 5 minutes, 100.6 ± 0.082 at 10 minutes, 100.13 ± 0.063 at 20 minutes and 100.16 ± 0.77 at 30 minutes.

Table 2

The percentage of the dissolved acetylcysteine in the two products in 1.2 pH buffer medium.

Sample No.	Test product Time (minutes)				Sample No.	Reference product Time (minutes)			
	5	10	20	30		5	10	20	30
1	100,01	100,12	100,08	100,31	1	100,07	100,14	100,22	100,32
2	99,84	100,05	100,10	100,24	2	99,87	99,92	100,08	100,14
3	99,87	100,08	100,12	100,47	3	99,92	99,95	100,06	100,09
4	99,92	100,11	99,98	100,12	4	100,14	100,09	100,14	100,13
5	100,02	100,09	99,97	100,27	5	100,05	100,12	100,17	100,19
6	99,98	100,14	100,05	100,15	6	100,07	100,11	100,15	100,24
7	99,48	99,95	100,14	100,32	7	100,15	100,17	100,21	100,23
8	99,72	99,97	100,21	100,45	8	99,89	99,95	100,02	100,07
9	100,03	100,05	100,18	100,37	9	100,08	100,06	100,08	100,12
10	99,85	99,90	100,05	100,09	10	99,97	100,04	100,07	100,09
11	99,74	99,72	100,02	100,19	11	100,02	100,07	100,14	100,11
12	99,35	99,87	99,98	100,21	12	100,05	100,12	100,16	100,23
Mean	**99,82**	**100,00**	**100,07**	**100,27**	**Mean**	**100,02**	**100,06**	**100,13**	**100,16**
Sd	*0,216*	*0,126*	*0,080*	*0,123*	*Sd*	*0,092*	*0,082*	*0,063*	*0,077*

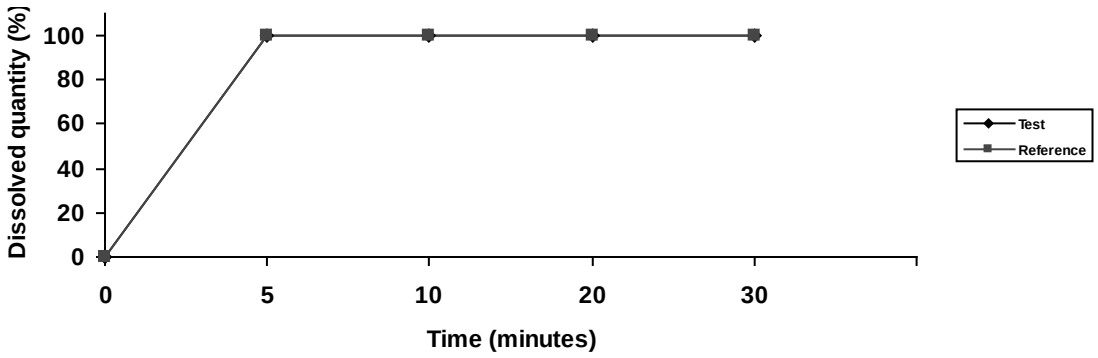

Fig. 1 Acetylcysteine dissolution profiles of the test and reference products dissolutions in 1.2 pH medium

As shown in fig. 1, the graphical representation of the mean concentrations of the 12 samples per sampling times, and the acetylcysteine dissolution profiles of the two products, 1.2 pH, also shows similarity between the test and reference formulation.

Dilutions in medium with 4.6 pH gave a higher uniformity of the mean acetylcysteine concentration percentage values of the two products (table 3, fig. 2). Thus, the in test product following values were determined: 99.79±0.150 at 5 minutes, 99.94±0.057 at 10 minutes and 100.00±0.040 at 20 minutes and 100.06 ±0.033 at 30 minutes. The same situation was found in case of the reference product, registering similar values: 99.40±0.581 at 5 minutes, 99.95±0.124 at 10 minutes, 100.03±0.061 at 20 minutes and 100.11± 0.068 respectively 30 minutes.

Graphical representation of acetylcysteine dissolution profiles in 4.6 pH medium, represented by the mean concentrations of the 12 samples corresponding to the sampling times, also indicating the similarity between the test product with the reference product (fig. 2).

Similar results were obtained for environmental and dilution with 6.8 pH (table 4, fig. 3). For the mean acetylcysteine concentrations percentage were 99.87± 0.803 at 5 minutes, 100.33±0.700 at 10 minutes, 100.60±0.373 at 20 minutes and 100.96±1.038 at 30 minutes for the test product. In case of the reference product the concentrations percentage were 95.78±

1.246 at 5 minutes, 99.89±0.869 at 10 minutes, 100.07 ±0.409 at 20 minutes and 100.75±0.506 at 30 minutes.

Table 3

The percentage of the dissolved acetylcysteine in the two products in 4.6 ph buffer medium

Sample No.	Test product Time (minutes)				Sample No.	Reference product Time (minutes)			
	5	10	20	30		5	10	20	30
1	99,92	100,02	99,98	100,04	1	99,49	99,92	99,98	100,07
2	99,87	99,95	99,97	100,04	2	98,37	99,87	99,93	99,99
3	99,75	99,87	99,96	100,02	3	100,04	100,11	100,09	100,24
4	99,84	100,02	100,05	100,11	4	98,89	99,76	99,97	100,05
5	99,93	100,01	100,03	100,08	5	99,18	100,08	100,06	100,14
6	99,62	99,91	99,98	100,07	6	100,02	100,07	100,12	100,18
7	99,85	99,98	100,07	100,12	7	99,57	99,94	100,04	100,09
8	99,53	99,90	99,94	100,06	8	99,72	99,84	99,98	100,07
9	99,84	99,92	99,98	100,02	9	99,07	100,02	100,06	100,14
10	99,85	99,93	100,02	100,07	10	100,05	100,07	100,11	100,18
11	99,92	99,97	100,01	100,03	11	99,83	99,97	100,04	100,11
12	99,51	99,85	99,96	100,04	12	98,57	99,76	99,98	100,08
Mean	**99,79**	**99,94**	**100,00**	**100,06**	**Mean**	**99,40**	**99,95**	**100,03**	**100,11**
Sd	*0,150*	*0,057*	*0,040*	*0,033*	*Sd*	*0,581*	*0,124*	*0,061*	*0,068*

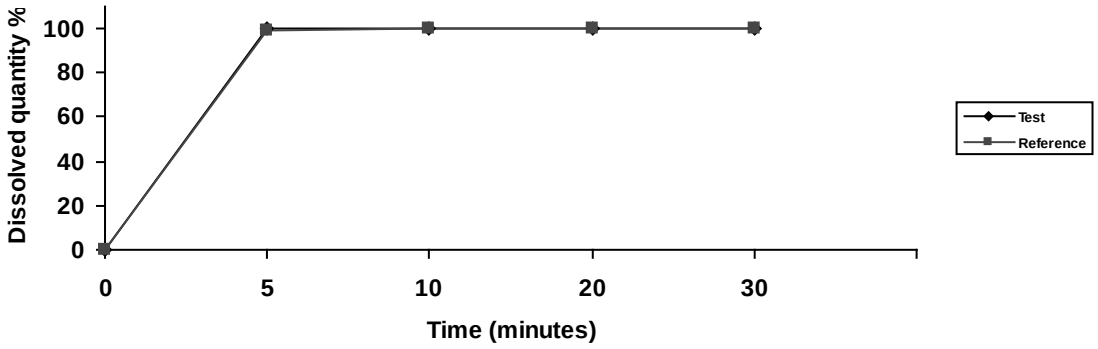

Fig. 2 Acetylcysteine dissolution profiles of the test and reference product in 4.6 pH dissolution medium

The similarity between the 2 products is also clear from the graphical representation of dissolution profiles in 6.8 pH medium of acetylcysteine, represented by mean concentrations of the 12 samples (fig. 3).

For bioequivalence of pharmaceutical products are generally used traditional processes based on in vivo tests Ognean et al., 2008). These experimental protocols often involve a single dose test. After the administration of a single dose of product the plasma concentrations of active substance are followed and pharmacokinetic parameters such as Tmax Cmax and AUC, are determined. These values are than compared with values obtained for the reference product. As a basic principle of these protocols compare the bioavailability of the active substance in the two products and establish equivalence relations between them (Hauck, Anderson, 1984). There is no consensus for the best measure of the rate of absorption for single-dose studies. (Smith, Brian, 2003).

Single dose administration of the active substance present the disadvantage that provides adequate accuracy only in mono-compartmental models, such as when administered intravenously. In addition the application of classical Wagner-Nelson method (Wagner and Nelson, 1964) to determine the ka is not indicated where it is expected that there are multi-compartmental models such as oral (Smith, Brian, 2003).

CONCLUSIONS

Average percentages in case of the dissolved 12 samples, obtained for test and reference products were more than 85% after 5 minutes in each of the 3

different pH dissolution media, indicating similar dissolution profiles making it unnecessary to calculate the similarity factor.

Based on in vitro dissolution profiles obtained the identical quantitative and qualitative composition (2 g acetylcysteine 6 g in powder) and the identical pharmaceutical formulation (oral powder) is not necessary for in vivo testing of the two investigated products.

The results obtained after testing in vitro of two acetylcysteine based mucolitic products confirms equivalence, according EMEA/CVMP/016/00-corr-FINAL (Guidelines for the Conduct of Bioequivalence Studies for Veterinary Medicinal Products).

Table 4

The percentage acetylcysteine dissolved in 6.8 pH buffer medium of the two products

Sample No.	Test product Time (minutes)				Sample No.	Reference product Time (minutes)			
	5	10	20	30		5	10	20	30
1	99,43	99,51	100,01	101,58	1	94,4	99,68	99,68	100,58
2	99,49	99,85	99,98	100,34	2	95,6	99,44	100,44	101,24
3	99,72	100,31	100,75	102,83	3	95,3	100,79	100,09	101,83
4	101,17	101,55	100,55	99,71	4	94,7	99,02	99,42	99,91
5	100,19	100,71	100,71	100,42	5	97,5	99,39	99,69	100,42
6	99,82	99,95	100,57	100,59	6	96,8	99,26	100,26	101,19
7	99,75	100,31	100,31	100,05	7	94,4	98,72	99,72	100,22
8	101,47	101,75	100,75	101,75	8	94,2	99,83	99,92	100,56
9	98,58	99,82	100,48	100,74	9	97,4	100,54	100,66	100,62
10	99,14	99,87	100,87	100,89	10	95,4	100,36	100,72	100,89
11	99,58	99,87	101,27	99,95	11	96,3	99,75	100,25	100,87
12	100,15	100,44	100,94	102,71	12	97,3	101,84	100,04	100,71
Mean	**99,87**	**100,33**	**100,60**	**100,96**	**Mean**	**95,78**	**99,89**	**100,07**	**100,75**
SD	*0,803*	*0,700*	*0,373*	*1,038*	*SD*	*1,246*	*0,869*	*0,409*	*0,506*

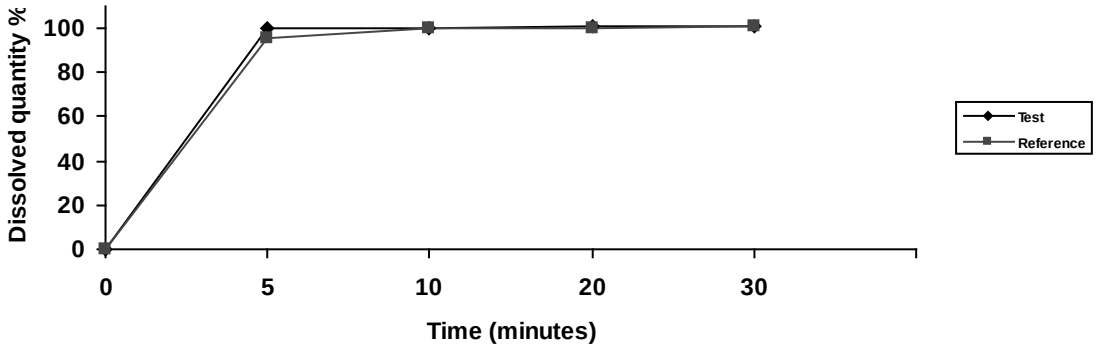

Fig. 3 Acetylcysteine dissolution profiles of the test and reference product in dissolution medium 6.8 pH

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