VALIDATION REPORT FLAME ATOMIC ABSORPTION SPECTROMETRY ASSAY FOR IRON DETERMINATION IN PHARMACEUTICAL PRODUCTS FOR VETERINARY USE

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abstract: The scope of the present study is to quantify iron in pharmaceutical products of veterinary use, using flame atomic absorption spectrometry (f_AAS). The determination is made after samples' mineralization in a $HCl:HNO_3$ (15:1, v:v) mixture followed by the instrument quantification and the validation of the result. The validation supposes evolution of liniarity, precision and accuracy of f_AAS method.

Introduction

The animals' health and performance depend on presence in administered a food of a lot of factors such as vitamins, vitamins with minerals or only minerals. The last have a special importance in an efficient and quick growing. The diet has an essential role in maintaining the animals' health and in diseases' prevention.

This concept is based on understanding the contribution of minerals in reducing the negative effects of free radicals and toxic metabolites towards immune processes of animals' organism. The key elements are: Cu, Zn, Mn, Fe şi Se [1÷4], which are added in animals' food through premixes and nutritive supplements.

The metals are administrated in food, so the organism can synthesize itself the enzymes, but in cases of their deficiency, oxidative stress and lesions of molecules and membranes could appear.

In Romania, the market of pharmaceutical products for veterinary use (premixes and nutritive supplements with vitamins, vitamins with minerals or only minerals) has increased in the last years. If in 1999 were proposed only 19 new products for authorization, in 2006 – the number of authorizations was 130. As a conclusion from the total new 644 pharmaceutical products authorized, 276 of them contain one of the trace elements (Fe, Cu, Zn and Mn named *oligo-elements*).

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Iron is an essential microelement for animal health and it is very important in oxygen biochemistry [5-7].

The purpose of this paper was to propose a flame atomic absorption spectrometric (f_AAS) method for iron determination in pharmaceutical preparations, such as premixes and nutritive supplements, where the matrix is very complicated.

Experimental

For quantification the sample is first calcinated and then the ash is solubilized by HCl [**8**], HF : H_2SO_4 : HCl (10:1:5, v:v:v) [**9**], HF:$HClO_4$ (20:25, v:v) [**10**] or HCl:HNO_3 (5:20, v:v) [**11**]. In the case of premixes and nutritive supplements, studied by us, the mixture HCl:HNO_3 (15:1, v:v) give the best results to determine Fe using f_AAS.

Reagents and apparatus

Iron standard stock solution (1000 µg Fe/mL), hydrochloric acid concentrate solution 12 mol/L (c = 1,19 g/mL), nitric acid 65% solution, (c = 1,42 g/mL) and hydrogen peroxide (c = 30%), (MERCK, Germany), ultrapure water (PURITE NEPTUNE ANALYTICAL – PURITE, England), hydrochloric acid solution 6 mol/L (prepared by us in our laboratory) and SA 37, powder of vitamins with minerals for veterinary use (INTERVET-The Netherlands), that contains iron under carbonates form (9,45 – 11,55 mg/g Fe).

A spectrometer GBC AVANTA (flame atomization) at 248.3 nm was used for determination.

Analitical procedure

About 2.0g powder of SA 37, exactly weighed, m_0, into chine crucible are calcinated at $450^0 \pm 20^0$C into a thermoadjustable oven (NABER THERM – GmbH Germany). The cold ash is moistened with care adding a few drops of hydrogen peroxide and keeps it on electric mantle, until H_2O_2 is decomposed. Over the ash add 15 mL of HCl solution 6 mol/L, evaporate the acid on electric mantle and then add 1 mL of HNO_3 concentrated solution. Add then 5 mL HCl solution 6 mol/L and continue the evaporation to dry. After cooling, the residue is filled to mark quantitative with tridistillated water in a 100 mL volumetric flask.

The Fe concentration is determined f_AAS by using the following equation:

$$\text{Total Fe (µg/g)} = c \times F_{dil} / m_0 \quad (1)$$

where: c = concentration of standard solution measured before sample (µg/mL)

F_{dil} = dilution factor,

m_0 = working quantity (g).

In the case of premixes and nutritive supplements, studied by us, the mixture HCl:HNO_3 (15:1, v:v) give the best results for Fe determination.

Results and discussion

The validation method for iron determination in pharmaceutical powders for veterinary use (premixes and nutritive supplements) through f_AAS must prove that the precedent equation include all the interferences that could affect the final result.

For the validation procedure the following parameters are evaluated: linearity, precision and accuracy. The precision represents the degree of concordance between the result and the reference value [12]. The systematically and random errors are quantified. The precision give us indications about the utility and applicability of this method to real samples.

Linearity is evaluated through graphical representation of the measured absorbance at λ = 248.3 nm. Fig. 1 shows the calibration curve and the regression line in the prediction range, as well as the trendline equation. Linearity shows the direct proportionality of the absorbance with Fe concentration solution into the given range (1.0 - 9.0 µg/mL).

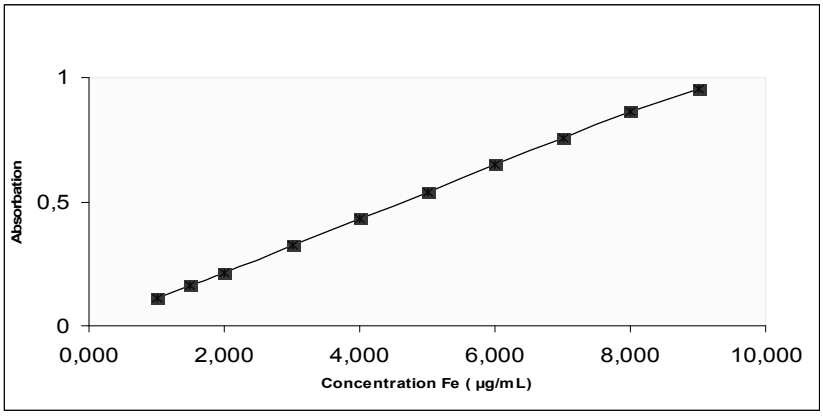

Fig. 1 *The calibration curve for Fe determination through f_AAS.*

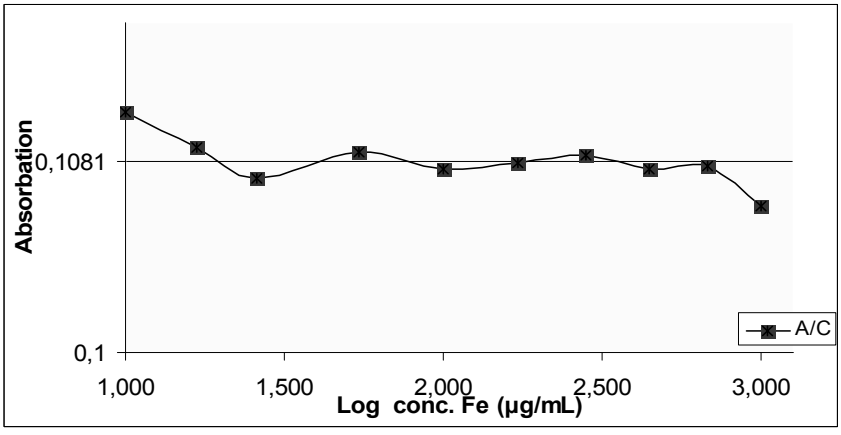

Fig. 2 *The graphical representation of the relative answers.*

The linear regression data are sometimes not sufficient for evaluation. An alternative approach is the ratio between the analyt response and it respective concentration to the iron content of the used standard solution and a graphical representation on a logarithmic scale, as can be seen in Fig. 2. The obtained line must be horizontal on the whole range, with a positive deviation at lower concentrations and a negative deviation at higher concentrations [13]. The deviations must not pass $\pm$ 5% [14], and in our study was -1.75 and +1,94%.

Another way of approach is represented by statistic test of linearity [15] that presumes the calculation using standard data for a linear calibration, as well as for non-linear function, too. The difference of dispersions DS^2 is calculated with the eqn. (2):

$$DS^2 = (N-2)s_{y1}{}^2 - (N-3)s_{y2}{}^2 \quad (2)$$

where N represent the number of levels of concentration, $s_{y1}{}^2$ is the standard residual deviation of one linearity regression (in our case y= 0.0040 + 0.107x, $s_{y1}{}^2 = 0.0000204$) and respectively $s_{y2}{}^2$ is the standard residual deviation of one non-linearity regression (in this case y= - 0,0033 + 0,1112 x - 0,0033 x^2, $s_{y2}{}^2 = 0.0000139$).

Necessary PG value for F test is calculated with eqn. (3):

$$PG = DS^2 / s_{y2}{}^2 \quad (3)$$

Comparing the obtained value, PG = 4.805, with theoretical F one (F= 5.35, from the table of Fisher–Snedecor's law) we can see that PG<F, so the non-linear function don't offer an improved adjustment for the calibration and we can use the linear calibration curve.

Table 1: Results concerning precision of method

Sample	Fe taken (mg/g)	Fe added (mg)	Fe found (mg/g)	Recovery (%)
I	10.51	1	11.505	99.50
		2	12.48	98.50
		3	13.48	99.00
II	10.55	1	11.545	99.50
		2	12.499	97.45
		3	13.51	98.66
III	10.54	1	11.55	101.00
		2	12.53	99.50
		3	13.55	100.33

ACCURACY, represents agreement for a measured value with the actual expected value [16]. Because in the pharmaceutical products studied by us it is impossible to obtain blanks that contain all components, for a better optimization of the results, after determination of the Fe content in SA 37 samples, we add 1 mL, 2 mL and respectively 3 mL standard solution of Fe (1mg/mL) to the sample, and after was re-evaluated the iron content.

The precision of method is described by the recovery of Fe content [17] in the sample. In this way we verify the efficiency of mineralization. The results obtained are presented in Table 1.

The acceptance criterion for the analyte's concentration of 1% must be between 97% and 103%.

PRECISION, represents the degree of agreement of a measured value with other values recorded at the same time, or in the same place or on similar instruments, also referred to as *repeatability* [**15**]. Iron determination from the samples SA 37 was realised on 6 determinations and the results are presented in Table 2. Precision is presented as standard deviation, relative standard deviation (RSD%) and confidentiality interval.

Table 2: Experimental results obtained for „repeatability"

Sample	Fe found (mg/g)	Average (mg/g)	Standard deviation	RSD %
1.	10.517	10.52	0.0109	0.13
2.	10.513			
3.	10.525			
4.	10.532			
5.	10.511			
6.	10.538			

Table 3: Experimental results obtained for intermediate precision

Parameter	Analyst I Average of 6 detns	Analyst II Average of 6 detns	Analyst III Average of 6 detns	Average of 12 detns	Average of 18 detns
Average (mg/g)	10.522	10.538	10.508	10.530	10.522
Standard deviation (mg/g)	0.0107	0.0106	0.0109	0.0131	0.0161
Standard average deviation (mg/g)	0.0044	0.0043	0.0044	0.0038	0.0038
CIIv	10,49-10,54	10,51-10,56	10,48-10,53	10,50 -10,55	10,48-10,55
$r/n^{1/2}$	0,0112	0,0111	0,0114	0,008	0,007
CIAv	10,51-10,53	10,52-10,54	10,49-10,51	10,52-10,53	10,51-10,53
Student „t" factor	2.57	2.57	2.57	2.20	2.11

Interval of confidence for individual values, (**CIIv**), (for 5 degrees of freedom and a probability of 95%) are determined with the formula:

$$\bar{x} \pm t^{n-1}{}_{5\%} \times s = 10{,}52 \pm 0{,}0279 \text{ mg/g} \quad (4)$$

Interval of confidence for average values, (**CIAv**), (for 5 degrees of freedom and a probability of 95%) are determined with the formula:

$$\bar{x} \pm \frac{t^{n-1}{}_{5\%} \times s}{\sqrt{n}} = 10.52 \pm 0{,}0114 \text{ mg/g} \quad (5)$$

Acceptance criterion of the method must be %RSD ≤ 2.7%.

INTERMEDIATE PRECISION: The analysis was made according to the described working procedure [18,19], by 3 analysts in 2 days. Table 3 presents the statistic results.

As can be seen the confidence interval is narrower, when using the average of 18 determinations.

Conclusions

The analytical results and the statistical evaluation of this step led to the following conclusion: the method is *LINEAR* in the range 1.0 – 9.0 µg/ml, *PRECISE* as can be seen from the RSD value (0.13%) and *EXACT*, resulting from recovery value. Once established this method it can be applied for all the pharmaceutical powders that contain iron.

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