

ATTACHMENT
No. 2

ROTTAPHARM			Quality Control Department
PRODUCT: ASSAY OF CRYSTALLINE GLUCOSAMINE SULFATE (CGS) IN THE SACHETS		OBJECT: Internal method	
IDENTIFICATION: MI069.1	Issued: 16 th Jan 2003	Valid from: 05 th Mar 2003	Revision date: 05 th Mar 2003
Reason of the revision: New validation			Page 1 of 7

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1 INTRODUCTION

The quantitation of the CGS in the sachets has been carried out with a potentiometric method employing NaOH as titrating agent in presence of a combined glass electrode.

2 EQUIPMENT AND REAGENTS

- Metrohm 682/686 Titroprocessor
- Metrohm 665 Dosimat
- Combined glass electrode
- Sodium hydroxide (about 0.1N)
- Demineralised water

3 TITRATION PARAMETERS

Titration mode: GET
 Stop volume: 20 ml
 Equivalent point criterium: 3

4 PROCEDURE

4.1 STANDARDIZATION OF NaOH (TITRATING AGENT)

The correct normality of the NaOH, employed in the potentiometric determination, is calculated by titrating a standard of anhydrous potassium hydrogen phthalate with the NaOH which normality is to be determined. For this purpose about 100 mg of standard, exactly weighed, are dissolved with 60 ml of water in a cell for potentiometric determinations and titrated with the NaOH about 0.1N whose normality must be exactly determined. This parameter is then calculated according to the following formula.

$$N = \frac{W}{EP * 204.23}$$

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where:

N = normality of NaOH solution
W = weight of standard anhydrous potassium hydrogen phthalate
EP = volume of NaOH required to get to the equivalence point
204.23 = molecular weight of potassium hydrogenous phthalate

4.2 SAMPLE PREPARATION

Transfer quantitatively the content of one sachet in a 200 ml volumetric flask. Dissolve the powder in water and bring to volume with the same solvent.

Transfer 25 ml of the resulting solution into a titration cell, dilute with about 25 ml of water and then titrate with NaOH in presence of a combined glass electrode.

Repeat the determination on two additional sachets. The content of CGS in the sachets will be calculated from the average of the three independent determinations.

5 CALCULATION

The amount of glucosamine sulfate (expressed as percent of the labelled amount in the sachets) is calculated for each determination according to the following formula.

$$\% = \frac{[Eq.2 - (Eq.1 * f)] * N * 286.655 * 3950 * 200}{W * 1884 * 25} * 100$$

where

Eq.2 is the second equivalent point, expressed in ml

Eq.1 is the first equivalent point, expressed in ml

286.655 is the equivalent weight of CGS

f is the correction factor relevant to the citric acid interference, i.e. 1.8 (see point 7.2, table 1)

N is the normality of the titrating agent

100 is the conversion factor to percent

1884 is the theoretical content of CGS in one sachet, expressed in mg

200/25 is a dilution factor

3950 is the theoretical net weight of one sachet, expressed in mg

W is the sample weight, expressed in mg

6 SPECIFICATION

The content of CGS in the sachets, with reference to the labelled amount, must lie between 95 and 105%.

7 VALIDATION OF THE METHOD

7.1 METHOD

The method is the potentiometric one, previously detailed.

7.2 CALCULATION OF THE CORRECTION FACTOR FOR CITRIC ACID INTERFERENCE

The formula to calculate the amount (Q) of CGS in the presence of citric acid is:

$$Q \text{ (CGS)} = [Eq2 - (Eq1 \times f)] \times N \times 286.655 \quad (1)$$

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The titration of the mix CGS/Citric acid gives rise to 2 equivalence points (Eq1 and Eq2). A correction factor, the "f" value, is introduced into the formula to take into account that the volume of titrating agent to be used to determine CGS does not correspond exactly to Eq2 - Eq1.

From a theoretical point of view, the two equivalence points are the result of the strong difference in the pK_a values among the three acidic groups of citric acid so that Eq1 only accounts for two carboxyl groups while the third one is included in the volume of titrating agent between Eq1 and Eq2.

The required correction factor "f" is deduced from a set of experimental determinations according to the formula:

$$f = \frac{1}{Eq1} \times [Eq2 - \{Q(CGS) / (N \times 286.655)\}] \quad (2)$$

where Eq1 and Eq2 have already been defined, Q(CGS) represents the true value of CGS, i.e. the known amount of CGS employed in the determinations, N is the normality of the titrating agent and 286.655 is the equivalent weight of CGS.

7.2.1 Determination of factor 'f'

To determine the 'f' value an experimental trial has been carried out by preparing and titrating, according to the method under evaluation, four mixtures, containing CGS, citric acid and the other excipients present in the sachets formulation in different ratios.

The detailed information about the way in which the four mixtures have been built-up can be found in table 1, while their composition and the relevant experimental values of 'f', calculated according to the formula (2), are described in table 2.

Table 1: mixtures statements

Component	Identification keys	Content (as % of the theoretical amount)
CGS	1	80
CGS	2	120
Citric Acid	A	50
Citric Acid	B	150
Other excipients	-	100

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Table 2: Determination of Factor f (Combined Citric Acid and CGS)

Sample	CGS mg/25 ml	Citric Acid weighed mg/200 ml	Eq.1 ml of titrating agent	Eq.2 ml of titrating agent	Calculated factor
1/A	191.07	12.79	0.145	6.872	1.4
			0.127	6.886	1.7
			0.117	6.909	2.1
1/B	190.88	38.27	0.406	7.395	2.1
			0.391	7.377	1.4
			0.406	7.359	2.2
2/A	285.93	12.38	0.127	10.237	1.8
			0.135	10.169	1.8
			0.111	10.222	1.7
2/B	286.56	38.44	0.464	10.734	1.6
			0.406	10.741	1.8
			0.361	10.730	2.0
				Mean	1.8
				S.D.	0.3
				±Conf. limit 95%	0.2

7.2.2 Conclusion

From the results reported in Table 2 an average factor f equal to 1.8 ± 0.2 has been calculated.

7.3 SPECIFICITY

To demonstrate the non-interference of the other excipients, two placebo mixtures, having the same composition as that of the sachets except for the citric acid, absent in one of two, were prepared and analysed according to the method under evaluation.

After the titration of the first mixture, without citric acid, no equivalent point was found, while from the titration of the second one, containing citric acid, an equivalent point was detected, corresponding to the quantity of citric acid present in the mixture, thus confirming the interference of this excipient.

Since this interference can be taken into account when calculating the assay of CGS (see point 7.2) and the other excipients do not interfere, we can conclude that the method is specific for the potentiometric determination of CGS in CGS sachets.

7.4 LINEARITY AND PROPORTIONALITY

For this purpose samples of CGS standard, ranging from about 1507 to 2260 mg, i.e. from the 80 to the 120% of the amount employed in the method under evaluation, have been tested in triplicate and the recovery subsequently determined.

The analytical results, together with the relevant statistical parameters, are listed in Table 3.

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Table 3: Experimental results

Standard conc. of CGS	Amount of CGS added (mg)	Amount of CGS found (mg)	Recovery %	Mean \pm confidence limits (p=0.05)
80%	190.85	190.78	99.96	99.93 0.49
	190.85	190.32	99.72	
	190.85	191.06	100.11	
90%	214.53	213.96	99.73	99.43 0.66
	214.53	213.09	99.33	
	214.53	212.88	99.23	
100%	238.53	239.00	100.20	99.96 0.56
	238.53	237.94	99.75	
	238.53	238.38	99.94	
110%	262.43	261.75	99.74	100.00 0.56
	262.43	262.84	100.16	
	262.43	262.70	100.10	
120%	286.09	287.73	100.57	100.31 0.57
	286.09	286.67	100.20	
	286.09	286.51	100.15	
Mean			99.93	
S.D.			0.35	
Confidence limits (p=0.05)			± 0.18	
% CV			0.35	

The parameters relevant to the regression between the true values (the added ones) and the corresponding recoveries (experimentally determined), calculated according to the least squares method, are listed in table 4.

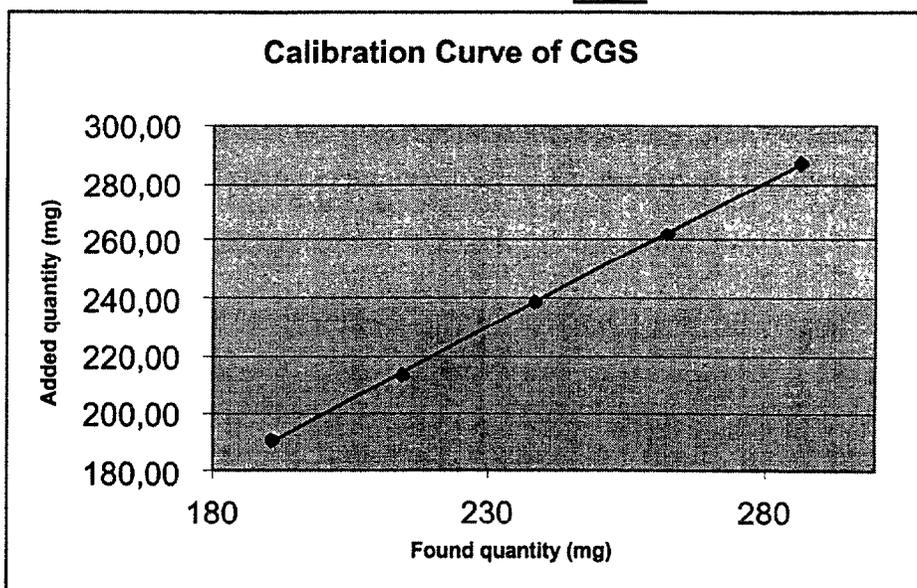
Table 4: experimental results

Regression parameters	Value
Intercept \pm conf. limits (p=0.05)	a=-3.36 \pm 4.43
Slope \pm conf. limits (p=0.05)	b=1.01 \pm 0.02
Correlation factor	r=0.9998
Student's t for 10 degrees of freedom (p=0.05)	t=2.23

A plot of the resulting regression line is shown in Fig.1

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Fig 1



From the plot of the regression line, from the very high value of the correlation coefficient (see table 4) and since the y-intercept of the regression is not significantly different from zero (see table 4), it can be argued that, at least in the tested range of values and under the specified operative conditions, the relationship between true and experimental values is proportional and linear.

7.5 ACCURACY

The test was carried out on a powder mix, reproducing the theoretical composition of the CGS sachets, obtained by thoroughly mixing a mixture of the excipients with standard CGS in the same ratio as that present in the sachets.

Six independent portions of the mix, exactly weighed, were analyzed according to the method under investigation and the corresponding recoveries determined.

The results, along with the relevant statistical parameters, are listed in Table 5.

Table 5: Accuracy

CGS added (mg)	CGS found (mg)	% recovery
238.78	237.71	99.65
239.78	239.38	100.25
238.78	236.50	99.04
238.78	237.00	99.25
238.78	238.70	99.96
238.78	237.82	99.60
Mean		99.61
S.D.		0.44
Confidence limits (p=0.05)		±0.47
%CV		0.44

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Since the confidence limits of the recovery include the 100% value and the CV is < 2%, the method can be considered sufficiently accurate.

The accuracy of the method can be furthermore assessed through the data employed to verify the proportionality and linearity of the method. In fact the confidence limits of each of average recovery values, listed in table 2, include the value 100%, thus confirming that the method is accurate for values ranging from 80% to 120% of the nominal amount of CGS employed.

7.6 PRECISION

The precision of the method has been verified carrying out ten independent determinations on samples of CGS sachets coming from the same batch.

The results and the relevant statistical parameters are set forth in Table 6.

Table 6: Precision

Analysis n.	Assay of CGS (from Glucosamine) (%)
1	98.69
2	98.97
3	98.08
4	98.72
5	98.91
6	98.42
7	98.45
8	97.97
9	97.84
10	98.26
Mean	98.43
S.D.	0.39
%CV	0.40

Since the resulting CV is < 2%, the method can be considered sufficiently precise to be used for the determination of crystalline glucosamine sulfate in CGS sachets

7.7 LIMIT OF DETECTION:

The method will be used only in the range from 80% to 120%. Therefore the limit of detection is not applicable.

7.8 LIMIT OF QUANTITATION:

The method will be used only in the range from 80% to 120%. Therefore the limit of detection is not applicable.

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1 INTRODUCTION

The quantitation of CGS in the tablets has been carried out with a potentiometric method employing sodium hydroxide as titrating agent in presence of a combined glass electrode.

2 EQUIPMENT AND REAGENTS

- Metrohm 682/686 Titroprocessor
- Metrohm 665 Dosimat
- Combined glass electrode
- Sodium hydroxide (about 0.1 N)
- Potassium hydrogen phthalate p.a., dried at 105°C
- Demineralised water

3 TITRATION PARAMETERS

Titration mode: GET
 Stop volume: 20 ml
 Equivalent point criterium: 3

4 PROCEDURE

4.1 STANDARDIZATION OF NaOH (TITRATING AGENT)

The correct normality of the NaOH, employed in the potentiometric determination, is calculated by titrating a standard of anhydrous potassium hydrogen phthalate with the NaOH whose normality is to be determined. For this purpose about 100 mg of standard, exactly weighed, are dissolved with 60 ml of water in a cell for potentiometric determinations and titrated with the NaOH about 0.1N whose normality must be exactly determined. This parameter is then calculated according to the following formula.

$$N = \frac{W}{EP * 204.23}$$

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where

W is the weight of the titrated standard, in mg

EP is the end point, in ml

204.23 is the molecular weight of potassium hydrogen phthalate.

4.2 SAMPLE PREPARATION

Accurately weigh 15 tablets of CGS and determine the average weight thereof. The tablets are then transferred in a suitable mortar and carefully ground.

About 400 mg, exactly weighed, of the resulting powder are transferred in a titration cell, diluted with 60 ml of water, allowed to stand under magnetic stirring for 15 minutes and then titrated with NaOH in presence of a combined glass electrode.

5 CALCULATION

The amount of CGS (expressed as percent of the labelled amount in the tablets) is calculated according to the following formula.

$$\% = \frac{EP * N * EW * MW}{W * QT} * 100$$

where

EP is the end point, in ml

N is the normality of the titrating agent

EW is the equivalent weight of CGS, i.e. 286.655

MW is the mean weight of the 15 tablets, in mg (see point 4.2)

W is the weight of the sample, in mg (see point 4.2)

QT is the theoretical quantity of CGS in one tablet, i.e. 942 mg

6 SPECIFICATION

The content of CGS in the tablets, with reference to the labelled amount, must lay between 95 and 105%.

7 VALIDATION OF THE METHOD

7.1 METHOD

The method is the potentiometric one, previously detailed.

7.2 SPECIFICITY

To demonstrate the non-interference of the excipients, a placebo mixture, having the same composition as that of the tablets, was prepared and analysed according to the method under evaluation.

As no end point was observed the lack of interference deriving from the excipients and therefore the specificity of the method are confirmed.

7.3 LINEARITY AND PROPORTIONALITY

For this purpose samples of CGS standard, ranging from about 267.0 to 400.5 mg, i.e. from the 80 to the 120% of the theoretical amount of CGS employed in the method under evaluation, have been tested in triplicate and the recovery subsequently determined.

The analytical results, together with the relevant statistical parameters, are listed in Table 1.

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Table 1: Experimental results

Standard conc. of CGS	Amount of CGS added (mg)	Amount of CGS found (mg)	Recovery (%)	Mean \pm confidence limits (p=0.05)
80%	268.51	267.49	99.62	100.06 0.97
	268.51	269.52	100.38	
	268.51	268.96	100.17	
90%	301.19	300.36	99.72	100.01 1.66
	301.19	303.51	100.77	
	301.19	299.78	99.53	
100%	334.58	333.68	99.73	99.89 1.53
	334.58	332.48	99.37	
	334.58	336.48	100.57	
110%	365.47	369.55	101.12	100.50 1.91
	365.47	368.20	100.75	
	365.47	364.14	99.64	
120%	405.39	404.36	99.75	99.77 1.04
	405.39	402.79	99.36	
	405.39	406.21	100.20	
Mean			100.05	
S.D.			0.56	
Confidence limits (p=0.05)			± 0.31	
% CV			0.56	

The parameters relevant to the regression between the true values (the added ones) and the corresponding recovery (experimentally determined), calculated according to the least squares method, are listed in table 2.

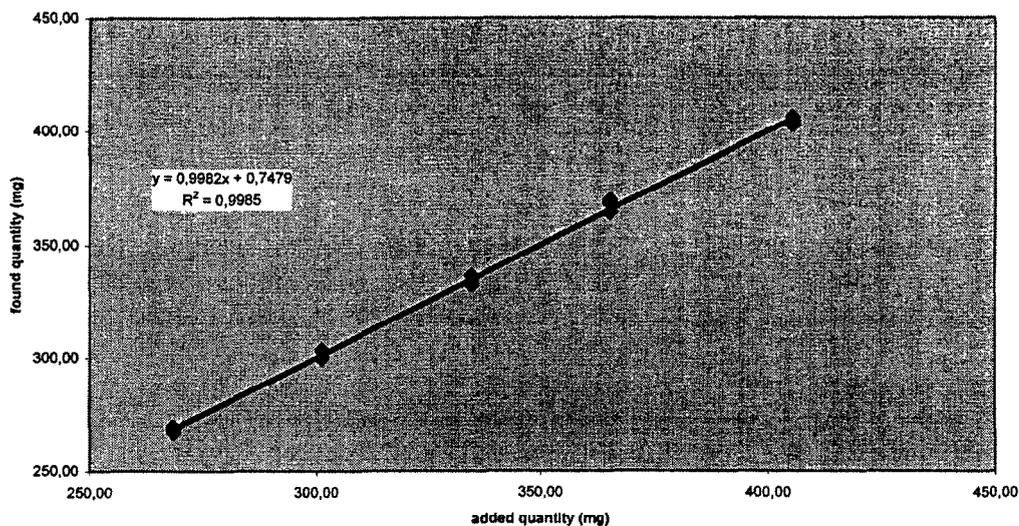
Table 2: Regression parameters

Intercept \pm conf. limits (p=0.05)	a=0.75 \pm 8.18
Slope \pm conf. limits (p=0.05)	b=0.998 \pm 0.024
Correlation coefficient	r=0.9992
Student's t for 10 degrees of freedom (p=0.05)	t=2.23

A plot of the resulting regression line is showed in the following figure.

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Calibration curve of CGS



From the plot of the regression line as well as from the very high value of the correlation coefficient (see table 2), it can be argued that, at least in the tested range of values and under the specified operative conditions, the relationship between true and recovery values is linear. Moreover, since the y-intercept of the regression is not significantly different from zero, the analytical method under investigation is also proportional (see table 2).

7.4 ACCURACY

The test was carried out on a powder mix, reproducing the theoretical composition of CGS tablets, obtained by thoroughly mixing the placebo, as mentioned on point 7.2, with standard CGS in the same ratio as that present in the tablets.

Six independent 400-mg portions of the powder mix were exactly weighed and analyzed according to the method under investigation. The corresponding recovery was then determined.

The results, along with the relevant statistical parameters, are listed in Table 3.

Table 3: Accuracy

CGS added (mg)	CGS found (mg)	% recovery
332.59	333.47	100.26
332.59	334.21	100.49
332.59	330.58	99.40
332.59	334.18	100.48
332.59	330.25	99.30
332.59	332.38	99.94
Mean		99.98
S.D.		0.53
Confidence limits (p=0.05)		±0.55
%CV		0.53

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Since the confidence limits of the recovery include the 100% value and the CV is < 2%, the method can be considered sufficiently accurate.

The accuracy of the method can be furthermore assessed through the data employed to verify the proportionality and linearity of the method. In fact the confidence limits of each one of the average recoveries listed in table 1, include the value 100%, thus confirming that the method is accurate within the range from 80% to 120% of the amount of analyte (CGS) employed in the analytical method.

7.5 PRECISION

The precision of the method has been verified carrying out ten independent determinations on samples of tablets coming from the same batch.

The results and the relevant statistical parameters are set forth in Table 4.

Table 4: Precision

Analysis n.	Assay of CGS (from Glucosamine) (%)
1	100.98
2	101.56
3	99.69
4	100.47
5	101.30
6	99.20
7	100.74
8	100.91
9	100.01
10	99.48
Mean	100.38
S.D.	0.83
%CV	0.83

Since, the resulting CV is < 2%, the method can be considered sufficiently precise to be used for the determination of CGS in the tablets.