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# FLUORESCENCE DENSITOMETRIC METHOD FOR THE DETERMINATION OF GLUCONIC AND LACTOBIONIC ACIDS ("SUGAR ACIDS") IN PHARMACEUTICAL PREPARATIONS

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## SUMMARY

An *in situ* fluorimetric method has been developed for the quantitation of gluconic and lactobionic acids and their salts in tablet formulations. The method is based on glycol cleavage with lead tetraacetate followed by treatment with dichlorofluorescein.

Calcium gluconate and lactobionate were determined in Calcium-Sandoz® and Ca-C 1000 Sandoz® effervescent tablets. The reproducibility corresponded to relative standard deviations between 0.7 and 3.5% (usually below 2%). Detection limits of 0.2 $\mu$g per spot can be obtained. Interfering compounds such as citric acid, sugars and ascorbic acid can be separated from the "sugar acids". The linearity of the calibration graphs between 0.5 and 5 $\mu$g per spot is satisfactory ($r = 0.994$–$0.999$). The method is simple and could be applied to the routine analysis of suitable pharmaceutical formulations. Other compounds with glycol structures should also be adaptable to this technique.

## INTRODUCTION

Several papers have appeared on the determination of "sugar acids". Lead tetraacetate[1], periodate[1,2] and copper salts[3] have been used in titrimetric techniques. Photometric techniques include cleavage with periodate followed by reaction with chromotropic acid[4]. Other methods are the reaction with *p*-nitrophenylhydrazine[5] or the reductive action of the "sugar acids" on iron(III) salts[6] or copper(II) salts[7]. Hilf and Castano[8] used a hydroxamic acid reaction for quantitation of "sugar acids". A polarographic method utilizing the molybdic acid complex[9] and an enzymatic technique based on gluconate dehydrogenase[10] have also been proposed. Some of these methods

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have been adopted following separation of the "sugar acids" on ion exchangers[2,11,12]. Paper chromatographic methods have also been used for the specific evaluation of mixtures of the calcium salts of "sugar acids"[13]. Most of these methods, however, are non-specific and not very sensitive, or they lack the precision necessary for quantitation.

It was the aim of this work to develop a method that does not have these disadvantages and that permits the determination of relatively low concentrations of "sugar acids" or their salts in complex pharmaceutical preparations. A reaction explored by Tanner and Duperrex[14] for the quantitation of sugar alcohols seemed most promising for this purpose. It involves the cleavage of "sugar acids" with lead tetraacetate on a thin-layer chromatographic (TLC) plate followed by spraying with dichlorofluorescein, which permits a direct fluorimetric determination.

## EXPERIMENTAL

### *Apparatus*

The fluorescence spectra were recorded with a Perkin-Elmer Model MPF-3 spectrofluorimeter, equipped with a TLC scanning attachment. A Zeiss PMQ II chromatogram spectrophotometer with a Servogor strip-chart recorder and a disc integrator were used for quantitative UV reflectance or fluorescence measurements. The Macherey-Nagel MN Sil G commercial glass plates were developed in Camag chambers.

### *Reagents*

The reagents were analytical grade throughout. The calcium salts of gluconic and lactobionic acid were obtained from Sandoz (Basle, Switzerland).

The following mobile phases were tested: I, isopropanol–ethyl acetate–water–concentrated ammonia (35:20:25:25, v/v); II, methanol–water–concentrated ammonia; (70:20:10, v/v); III, acetone–ethyl acetate–water–concentrated ammonia (50:20:20:10, v/v).

Two solutions were prepared for the reaction on the TLC plates: A, a 2% (w/v) solution of lead tetraacetate (Fluka, Buchs, Switzerland) in glacial acetic acid; B, a 1% (w/v) solution of 2,7-dichlorofluorescein in absolute ethanol. Volumes of 25 ml each of solutions A and B were mixed and diluted to 1 l with dry benzene. This dipping solution is stable for about 2 h.

### *General procedure*

The aqueous solutions of the cacium salts of the "sugar acids" are applied with 2-$\mu$l microcaps. The plates are dried for 1 h in air and then for 30 min in a vacuum drying oven at 100°.

The chromatographic development of the plate is carried out by the ascending technique in about 4 h over a distance of 15 cm. The plate is dried for 2 h in air and then for 30 min at 100° in a vacuum drying oven.

The fluorescence reaction is carried out by dipping the plate quickly into the dipping solution. The plate is kept in the dark for 30 min and then dried for 30 min at 50° in a vacuum drying oven. The plates are stored in the dark until measurements are carried out.

The experimental parameters for fluorescence measurement were the Pr-M setting on the Zeiss instrument operated with an St-41 mercury lamp, with excitation using an M 365 filter and emission with the monochromator set at 530 nm. For UV reflectance, the M-Pr setting was used and measurements were carried out at $\lambda_{max.} = 490$ nm. The calibration functions used were $A = a + bc$ for fluorescence and $A^2 = a' + b'c$ for reflectance measurements ($A$ = area of the recorded signal).

*Procedure for pharmaceutical preparations*

Effervescent tablets of Calcium-Sandoz® and Ca-C 1000 Sandoz® were analyzed.

*Calcium-Sandoz.* One tablet was dissolved in 1000 ml of water, which corresponds to about 2 $\mu$g of calcium gluconate and 2 $\mu$g of calcium lactobionate per 2-$\mu$l spot.

The calibration solutions were made with 100 mg of calcium gluconate and 100 mg of calcium lactobionate in 50 ml of water. From this solution, 4-, 5- and 6-ml portions were diluted to 10 ml to give concentrations of 1.6, 2.0 and 2.4 $\mu$g., respectively, per 2-$\mu$l spot, corresponding to 80, 100 and 120%, respectively, of the expected sample value ($S$).

Using the data-pair technique of Bethke *et al.*[15], 12 spots were applied according to the following scheme: $80\%/S_1/100\%/S_2/120\%/S_3/80\%/S_1/100\%/S_2/120\%/S_3$. The averages of the two corresponding readings were then taken for evaluation. The chromatographic and fluorescence generating steps have been described under *General procedure.*

*Ca-C 1000 Sandoz.* One tablet was dissolved in 500 ml of water, which corresponds to about 2 $\mu$g of calcium gluconate per 2-$\mu$l spot.

## RESULTS AND DISCUSSION

*Chromatography*

The $R_F$ values for the three solvent systems tested, are given in Table I.

TABLE I

$R_F$ VALUES FOR THE THREE SOLVENTS SYSTEMS

| *Calcium salt* | *Solvent system* | | |
|---|---|---|---|
| | *I* | *II* | *III* |
| Gluconate | 0.32 | 0.80 | 0.23 |
| Lactobionate | 0.20 | 0.65 | 0.10 |

Solvent system I was chosen for further work as it gave the best separation of the "sugar acids" from interfering sugars, citric acid and vitamin C in the pharmaceutical formulations (see Figs. 1–3). Of several commercial plate types tested, Macherey-Nagel MN Sil G gave the best results. On Merck plates, a complete separation was difficult.

*Reaction and spectra*

The fluorescence spectrum measured directly on the TLC plate is shown in Fig. 4, with excitation maxima at 468 and 490 nm and emission at 530 nm.

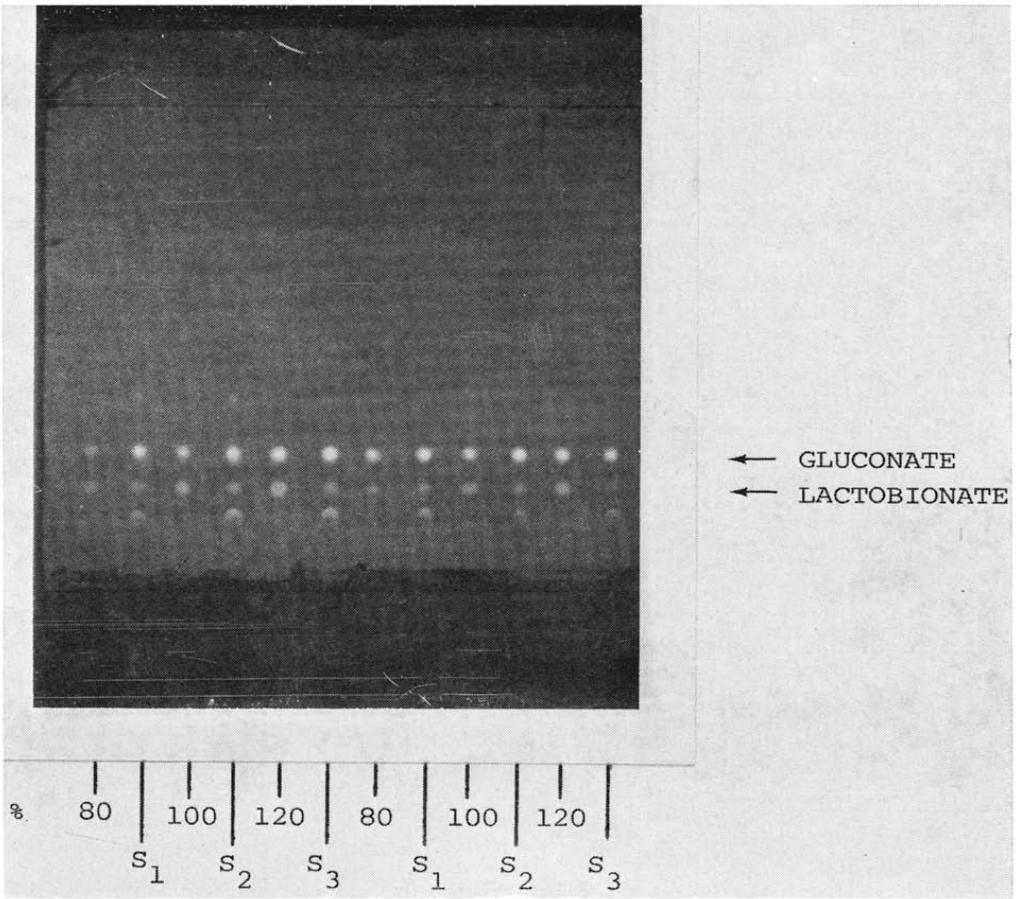


Fig. 1. Actual chromatogram for the analysis of a Calcium-Sandoz tablet.

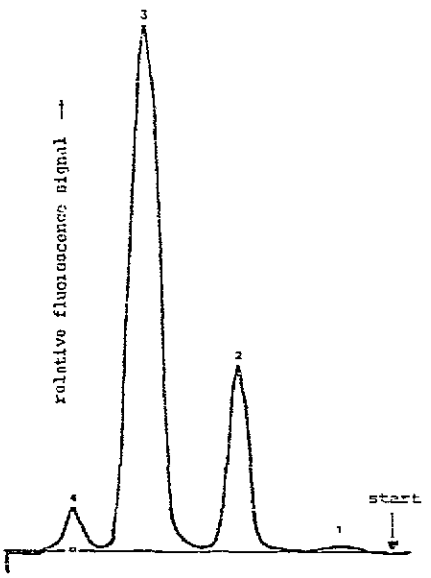


Fig. 2. Scan of a sample spot of the chromatogram in Fig. 1. 1 = Citric acid; 2 = lactobionate; 3 = gluconate; 4 = sugar.

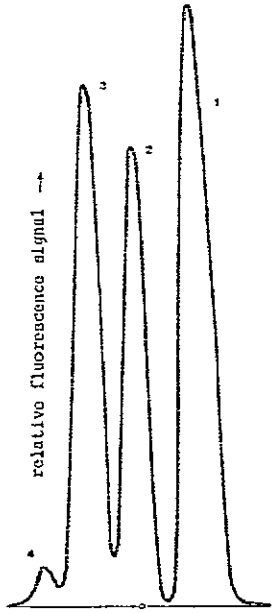


Fig. 3. Scan of a sample spot of a Ca-C 1000 Sandoz tablet. 1 = Gluconate; 2 = sugar; 3 = ascorbic acid; 4 = lactate.

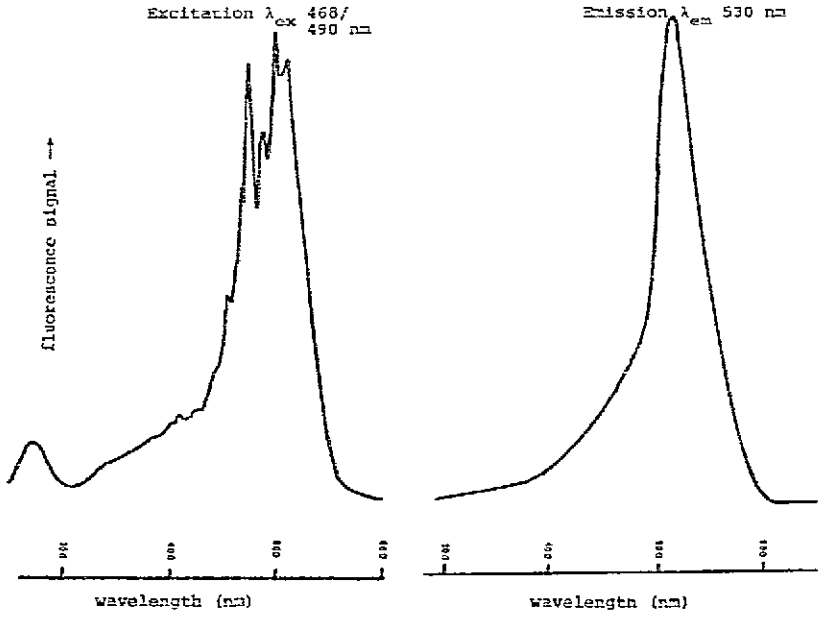


Fig. 4. Excitation and emission maxima of the fluorescence spectrum recorded directly on the "sugar acid" spots on a TLC plate after treatment with lead tetraacetate and dichlorofluorescein.

The reaction is believed to be based on a glycol cleavage of the "sugar acid" by means of lead tetraacetate. The dichlorofluorescein is irreversibly converted into a non-fluorescent oxidation product, which causes complete quenching of the background. On the spots containing the "sugar acids", part of the dichlorofluorescein remains undisturbed as the lead tetraacetate has reacted with the "sugar acid". This causes a fluorescence, the intensity of which is proportional to the concentration of "sugar acid". The fluorescence spectrum presented in Fig. 4 has been shown to be identical with the spectrum of dichlorofluorescein.

The dichlorofluorescein spot has a yellow-orange colour that can also be measured by reflectance measurements at 490 nm.

*Stability of the fluorescence*

The fluorescence behaviour is essentially the same as that observed for dichlorofluorescein. The intensity increases rapidly during the first 30 min if the plates are kept in air following the dipping procedure. The drying step (30 min at 50° under vacuum) causes the fluorescence to become stable for several days if the plates are stored in the dark.

Repetitive scanning and prolonged UV irradiation of the spots in the $\lambda_{ex}$ region (1 h) does not cause a significant variation in fluorescence intensity. Hence the stability is excellent for analytical purposes.

*Quantitative data*

*Analysis of drug substances.* The detection limits measured at a 3:1 signal-to-noise ratio (95% confidence limit) are 0.2 $\mu$g per spot with fluorescence and approximately 1 $\mu$g per spot with reflectance measurements. The reproducibility of the method is limited by the reaction, even though this influence can be kept low by using the dipping technique. Utilizing the data-pair technique[15], it is possible to obtain reproducibilities corresponding to a relative standard deviation between 0.7 and 2.2% for the fluorescence measurements and between 1.5 and 7.0% for reflectance measurements at 490 nm. The tests were carried out on three plates with ten spots each with concentrations between 2 and 2.5 $\mu$g. These values could probably be improved by using an electronic integrator.

The calibration range from 0.5 to 5 $\mu$g was investigated for fluorescence, using the function $A = a + bc$. The correlation coefficients were between 0.994 and 0.999. For reflectance measurements, the correlations were not as good.

*Analysis of tablets.* It can be seen from Figs. 1–3 that a clean separation of the "sugar acids" from interfering components is possible with solvent system I. The separation takes about 4 h. The reproducibility of the actual assays is only slightly inferior in comparison with that for the drug substance itself, and may be as high as a relative standard deviation of 3.5%.

The accuracy was tested on samples of three different batches of Calcium-Sandoz and Ca-C 1000 Sandoz. The results were well within the tolerance limits. For calcium gluconate the theoretical value was 16.75% and the experimental value 16.92% for Calcium Sandoz and the theoretical value was 8.20% and the experimental value 8.75% for Ca-C 1000 Sandoz. For calcium lactobionate, the theoretical value was 16.90% and the experimental value 15.20% (Calcium Sandoz). The values for calcium

lactobionate were rather low. Good agreement was found between the values obtained by the reflectance and fluorescence techniques.

## CONCLUSIONS

This *in situ* fluorimetric method is suitable for the routine analysis of gluconic and lactobionic acids in pharmaceutical formulations. It combines the required features of sensitivity, specificity, accuracy and simplicity to a sufficient degree to make it superior to previously used techniques.

The same analysis can be carried out by evaluation of the chromatograms by reflectance measurements at 490 nm. The quantitative data are inferior in this approach, probably due to the lower sensitivity of the reflectance signal (poorer signal-to-noise ratio).

The routine analysis of the pharmaceutical products tested can be carried out easily in 1 day, and most of this time is used for development and drying. The same method should be applicable to other compounds with a glycol structure provided that the ratio of dichlorofluorescein to lead tetraacetate in the dipping solution is adjusted as appropriate.

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