Journal of Chromatography, 79 (1973) 223–228

CHROM. 6596

A QUANTITATIVE ASSAY FOR SENNOSIDES BY FLUORIGENIC DERIVATIZATION ON THIN-LAYER CHROMATOGRAMS

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(Received December 14th, 1972)

SUMMARY

The analysis of the laxatives, sennosides A, B and C, is carried out after separation by thin-layer chromatography. The fluorescing derivatives are formed by spraying the developed chromatoplates with a solution of hydrazine, adjusted to pH 8 with HCl. The spots are determined by *in situ* fluorimetry with a Zeiss chromatogram spectrophotometer in the fluorescence mode. Reactions involved in the derivative formation are suggested and the stability of the derivatives on silica gel layers is examined. Calibration curves are linear up to 800–1000 ng per spot. Detection limits between 1–5 ng per spot are observed. The analysis of commercially available preparations is reported, with no interference from co-extractives. The technique is simple, sensitive and rapid, and should be applicable to other anthraquinone compounds.

INTRODUCTION

Sennosides A, B and C are of considerable pharmaceutical interest owing to their laxative properties. The analytical methods which are currently available, however, leave much to be desired in terms of their efficiency, sensitivity and ease of application. The most commonly used approach involves the formation of colored derivatives followed by absorption spectrophotometry[1–4]. This procedure gives only a total sennoside content, and in order to distinguish between the A, B and C isomers a good preliminary chromatographic separation is needed, followed by a method of evaluation that is possibly more sensitive than one based on natural UV absorption or formation of colored species[5,6].

In this work, the potential of a thin-layer chromatographic (TLC) separation in conjunction with a rapid *in situ* fluorimetric approach via derivatization was investigated in order to ensure both specificity and sensitivity of the resulting technique.

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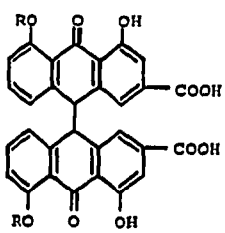
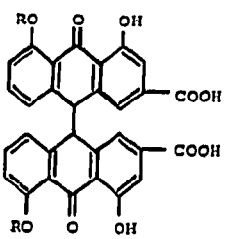
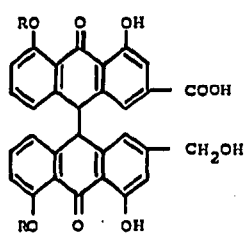
EXPERIMENTAL

Reagents

Sennosides A, B and C (Table I) were obtained from Sandoz Ltd., Basle, Switzerland. Solutions of these compounds were prepared at concentrations of 0.1 mg/ml in methanol containing one drop of triethylamine per 100 ml. These stock solutions were diluted as required. The spray solution consisted of 65 % aqueous hydrazine (Fisher Scientific Co.) carefully adjusted to pH 8 by the dropwise addition of 6 *N* HCl.

TABLE I

STRUCTURES OF SENNOSIDES

Compound	*Structure*[a]	
Sennoside A		(optically active)
Sennoside B		(*meso form*)
Sennoside C		

[a] R = D-glucose.

Chromatographic separations were carried out on glass plates coated with Silica Gel G (Macherey Nagel, Düren, G.F.R.) according to manufacturer's directions. Commercially prepared silica gel sheets (Eastman No. 6061) were also used. The solvent system found to be most suitable consisted of ammonia solution–ethanol–isopropanol–ethyl acetate (35:10:20:25).

General procedure

The sennosides were spotted with a 10-μl Hamilton microsyringe *ca.* 1 cm from the bottom of the plate and dried. The chromatograms were developed over a distance of 10 cm and dried under a stream of warm air for about 5 min. The plates were then sprayed with hydrazine solution until they became translucent, placed horizontally for 1 min for complete reaction to take place and then dried under a stream of warm air. The spots were made visible under a Camag universal UV lamp at 350 nm. The fluorescence spectra of the derivatives were recorded *in situ* on an Aminco-Bowman spectrofluorimeter equipped with a TLC scanning accessory. Quantitative measurements were carried out with a Zeiss chromatogram spectrophotometer modified for fluorescence measurements. The chromatographic peaks were integrated with an Autolab Vidar digital integrator.

Extraction of sennoside tablets

One tablet of Sennokot® (Purdue, Frederik Canada Ltd.) was placed in a 100-ml Pyrex calibrated flask and 50 ml of distilled water containing 5 drops of triethylamine were added. The contents were heated at 80° for 20 min with frequent shaking. After cooling the flask to room temperature, methanol was added to bring the volume to 100 ml. For chromatography, 5-μl aliquots of this solution were spotted along with standards. For comparison, ten tablets from the same package were analyzed.

RESULTS AND DISCUSSION

Chromatography

Good separation of the three isomers was achieved on home-made plates and commercial sheets with the above solvent system. The order of separation on the plates was sennoside C (R_F 0.52), sennoside A (R_F 0.39) and sennoside B (R_F 0.19). The same order but with poorer separation was observed on the sheets.

Spray reaction

The reaction of the 65 % aqueous hydrazine spray with the sennosides on the plate was instantaneous, but was slower with more dilute solutions such as 10 %, with which 60 sec were required for completion of the reaction. The most important step was the adjustment of the pH of the spray to *ca.* 8, as mentioned above. Under neutral or acidic conditions no fluorescence was produced. Other bases, such as ammonia, NaOH, alkylamines and phenylhydrazine, were also tested for possible reactions but no fluorescence was produced even after 30 min at 70°.

A possible reaction scheme is illustrated in Fig. 1. In step 1 the hydrazone of the sennoside is formed, which then undergoes an elimination–addition reaction (step 2). The resulting highly fluorescing molecule exhibits enhanced aromaticity, which is of the nature of a substituted anthracene derivative.

Fluorescence properties

Fig. 2 shows the stability of fluorescence as a function of time for sennosides A and B sprayed with different solutions. It can be seen that the fluorescence decreases drastically when a 65 % aqueous solution of hydrazine hydrate is used. With a 10 % solution, an almost equally sharp decrease is observed until a plateau at about 25 %

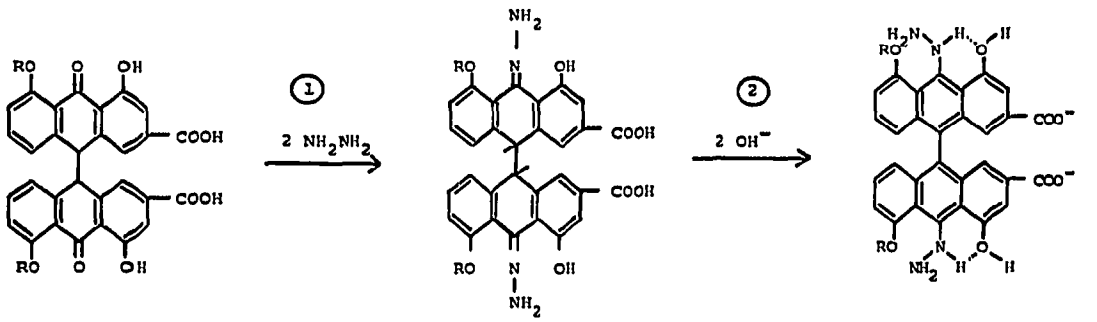


Fig. 1. Suggested reaction scheme for the formation of the fluorescent sennoside derivatives. 1 = formation of the hydrazone; 2 = increasing the aromaticity of the ring systems to form a fluorescent product.

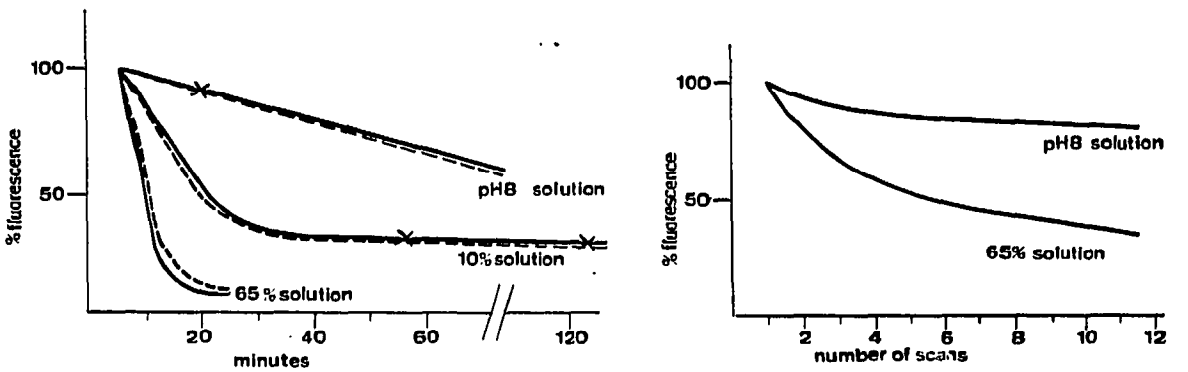


Fig. 2. Time study of the various spray reactions examined. – – – – –, sennoside A; ——— sennoside B.

Fig. 3. Effect of repeated scanning of the sennoside derivatives with a Zeiss chromatogram scanner.

of the original fluorescence is reached; this spray composition could therefore still be useful. However, when a 65 % hydrazine solution adjusted to pH 8 (see EXPERIMENTAL section) is used as the spray reagent, a reasonably stable and high fluorescence is produced. The loss of fluorescence could be attributed in part to the excess of base present after derivative formation; this effect is minimized at pH 8. In addition, the stability of the derivatives seems to be reduced when they are adsorbed on silica gel, in comparison with solution conditions.

The effect of UV irradiation due to repetitive scanning on the fluorescence of the derivatives is demonstrated in Fig. 3. The 65 % hydrazine spray, adjusted to pH 8, again produces more stable fluorescence. As one or two scans are usually sufficient for a complete analysis, the decomposition due to the measurement process can be considered to be negligible. The fluorescence spectra were, as expected, essentially identical for all three derivatives. A typical spectrum is shown in Fig. 4. The excitation and emission maxima are more than 150 nm apart, which would permit simple filter fluor-

imeters to be used. An additional advantage is the position of the spectrum at long wavelengths. Naturally fluorescing interferences, for example in crude plant extracts or biological samples, tend to fluoresce at lower wavelengths, so the method could be fairly specific.

For quantitative analysis with the Zeiss instrument, a 436-nm excitation filter was used and the fluorescence emission was measured with a 555-nm monochromator setting. The emission intensity was about 2.2 times higher with this filter than with the 365-nm filter, as expected from the absorption spectrum.

Analytical data

Instrumental detection limits ranged between 1 and 5 ng per spot (3 : 1 signal-to-noise ratio) for the three sennoside isomers. Linear calibration curves were obtained up to 800–1000 ng per spot. The reproducibility of the method at concentrations greater than 40 ng per spot was about ± 3 % relative standard deviation, and we believe that this could be further improved.

The analysis of ten individually assayed commercial tablets gave the following results: sennoside A, 4.00 mg; sennoside B, 4.13 mg; and sennoside C, 0.71 mg. These values correspond to a total concentration of sennosides of 8.84 mg per tablet. The amount declared on the package was 8.70 mg of sennoside A and B per tablet. Fig. 5 shows a typical chromatogram and the corresponding scan for the tablet analysis.

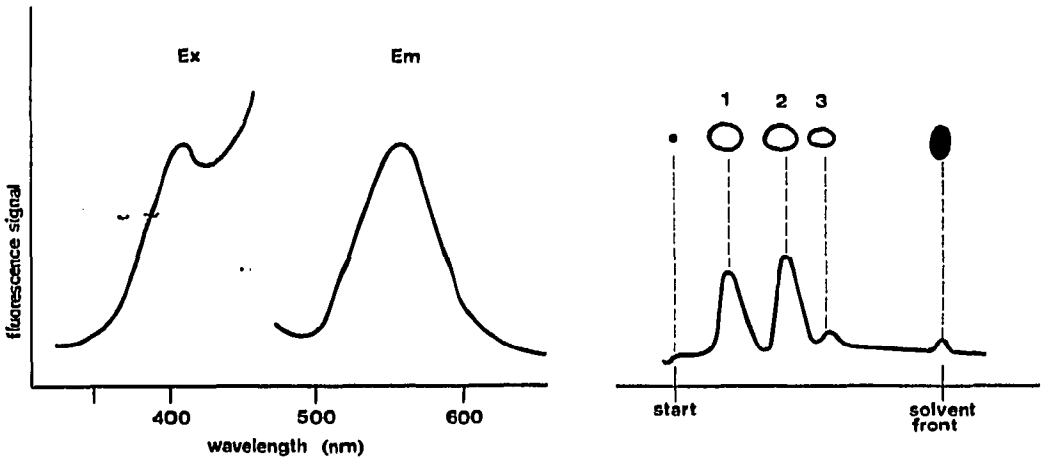


Fig. 4. Fluorescence spectrum of the derivative of sennoside A recorded with an Aminco-Bowman spectrophotofluorimeter with a thin-layer scanning accessory. Ex = excitation, Em = emission.

Fig. 5. Sketch of thin-layer chromatogram and the corresponding chromatogram scan obtained with the Zeiss instrument on a Sennokot tablet. 1 = sennoside B; 2 = sennoside A; 3 = sennoside C.

CONCLUSIONS

The method described offers a simple and rapid approach to the analysis of sennosides A, B and C in simple pharmaceutical preparations. It should also have potential for the analysis of *Sennae* leaf extracts and possibly biological samples. An

extension of this method to other anthraquinone compounds could be of interest. Owing to the rapid reaction that also occurs in solution, a similar approach could be used for the detection of sennosides after high-speed liquid chromatographic separation. Mixing chambers for reagent and compounds prior to entering a detector have been constructed[7,8].

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Research Council of Canada. The authors are grateful to Sandoz Ltd. for providing the sennosides.

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