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Note

Quantitative determination of myo-inositol in pharmaceutical preparations and organic extracts by high-performance thin-layer chromatography using fluorescence

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Inositol (hexahydroxycyclohexane) is an isomer of glucose. There are seven optically inactive and one pair of optically active stereoisomeric forms of inositol possible, of which only one, the optically inactive myo-inositol, is nutritionally active[1]. Myo-inositol occurs widely, both in the free state and in combined form (in phytin, phospholipids, etc.). The compound is assigned to the group of B vitamins. Myo-inositol is used in the treatment of hepatopathy and progressive muscular dystrophy (with vitamin E and choline)[2] and acts as a lipotropic agent.

Several chromatographic techniques have been developed for the analysis of inositol. Paper chromatography (PC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC) have all been reported. The separation by GLC requires pre-column derivatization[3,4]. The sensitivity of detection is poor for HPLC with refractive index detection[5]. PC and TLC of myo-inositol have been described by several workers[6–14], who considered only its separation and identification, not its determination. Radioactive inositol has been assayed on silica gel glass-fibre sheets[15]. The spectrophotometric determination of myo-inositol has been combined with separation by TLC[16]. Microbiological determinations have also been proposed[17].

This paper describes an *in situ* fluorimetric high-performance thin-layer chromatographic (HPTLC) method for the quantitation of free myo-inositol. The fluorescence detection is based on a reaction described by Tanner and Duperrex[18–20]. The assumed mechanism of this reaction has been reported[21] and it has been utilized by several workers[21–23]. Lead tetraacetate has been employed for the oxidation of inositols. The acids corresponding to the dialdehydes obtained by breaking the cyclitol ring have been isolated and identified[25].

In this work we used a modification of the detection method with lead tetraacetate as the oxidizing agent and sodium fluorescein as the fluorescent dye in dichloromethane. A low detection limit of myo-inositol was achieved, *viz.*, *ca.* 0.5 ng per spot. The stability of the dipping solution was improved also. The fluorescence of the spots on the plate is stable for several weeks if it is stored in a closed, opaque container. The suggested solvent system permits the separation of myo-inositol from interfering components in pharmaceutical preparations such as sugars.

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EXPERIMENTAL

All solvents were of analytical-reagent grade. Lead tetraacetate and sodium fluorescein were purchased from Merck (Darmstadt, G.F.R.). Myo-inositol, epi-inositol, myo-inositol 2-monophosphate (dimonocyclohexylammonium salt) and phytic acid (sodium salt) were supplied by Sigma (Munich, G.F.R.). Prohepar®-dragées, Prohepar syrup and the organic extracts were obtained from Nordmark-Werke (Uetersen, G.F.R.). Proheparum®-Gold dragées and Prohep® tablets were manufactured by Hanil (Seoul, Korea) and by Noristan (Pretoria, South Africa), respectively.

A stock standard solution of myo-inositol was prepared by dissolving 100 mg of substance in water and diluting to 100 ml. This solution was diluted to give the concentrations required for three- or five-point calibration graphs.

Twenty dragées or tablets were pulverized and the amount corresponding to one dragée or two tablets was weighed into a 50-ml volumetric flask and extracted with 50 ml of water for 30 min in a mechanical shaker. The mixture was filtered and diluted with water to 100 ml. A 2.5-ml volume of Prohepar syrup and 2–3 g of organic extracts (dry weight) were treated in the same way. One dragée and 2.5 ml of syrup contain 25 mg of myo-inositol; one tablet contains 12.5 mg of myo-inositol. The organic extracts (dry weight) contain *ca.* 0.4–0.6% of free myo-inositol.

Two solutions were prepared for the fluorescence detection: solution 1, a 3% (w/v) solution of lead tetraacetate in glacial acetic acid; solution 2, a 1% (w/v) solution of sodium fluorescein in *ca.* 95% ethanol. A 5-ml volume of solution 1 and 2.5 ml of solution 2 were mixed successively with 100 ml of dichloromethane.

The separations were performed on 10 × 10 and 10 × 20 cm pre-coated silica gel 60 HPTLC plates without fluorescent indicator (Merck).

The solutions were applied on the plates using a 100-nl platinum–iridium capillary (Antech, Bad Dürkheim, G.F.R.) and a Nano-Applicator (Camag, Muttenz, Switzerland) in a line 4 mm apart, using the data-pair technique[25].

The plates were developed in unsaturated chromatographic chambers (Camag, HPTLC twin-through chambers) at room temperature in darkness, using acetone–methanol–water (2:2:1) as the solvent. The migration distance was 3–5 cm and the separation time was *ca.* 10–20 min. After development the plates were dried and dipped into the dipping solution (Desaga dipping chambers). The plates were kept in darkness for 30 min and then dried in a stream of air for *ca.* 60 min. The plates were stored in darkness.

The separated spots were scanned with a KM-3 chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) using the microoptical accessory. The Zeiss instrument was attached to a KM-3 Automatik (Eckelmann, Wiesbaden, G.F.R.) for the evaluation and automatic positioning of the spots in the measuring slit. Servogor 310 recorder was used (BBC, Goerz, Austria). The scanning speed in the direction of chromatography was set at 30 mm/min, with a recorder speed of 30–50 mm/min. The conditions for the fluorescence measurements were: monochromator–sample set-up, excitation wavelength 436 nm (St 41 mercury lamp), fluorescence wavelength >460 nm (F1-46 cut-off filter). The monochromator slit length was 1–2 mm and the slit width was 0.10–0.15 mm (microoptics). The emission fluorescence spectrum was measured with the sample–monochromator set-up (St 41 mercury lamp, M 436 filter).

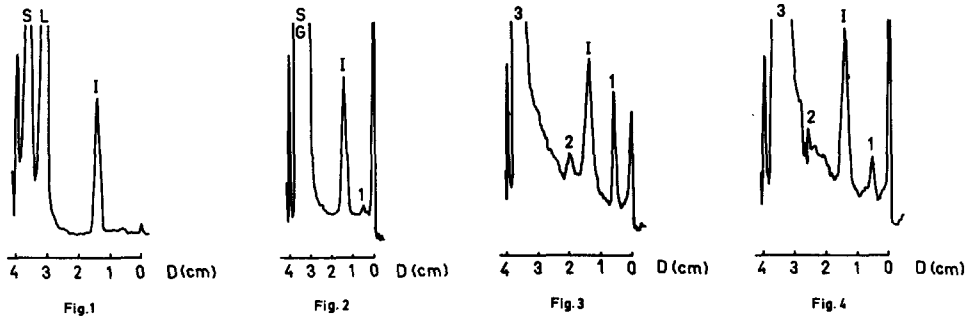

Fig. 1. Densitogram of Prohepar dragée sample. Adsorbent: HPTLC silica gel 60 without fluorescent indicator (Merck). Solvent system: acetone–methanol–water (2:2:1). Application volume: 100 nl. Separation distance: 4 cm. Spectrofluorimetric measurement: monochromator–sample set-up, microoptics, excitation wavelength 436 nm (St 41 mercury lamp), fluorescence wavelength > 460 nm (F1-46 cut-off filter). *D* = distance migrated. I = Myo-inositol; L = lactose; S = saccharose.

Fig. 2. Densitogram of Prohepar syrup sample. Conditions as in Fig. 1. I = Myo-inositol; S = saccharose; G = glucose; 1 = unknown.

Fig. 3. Densitogram of beef liver extract sample. Conditions as in Fig. 1. I = Myo-inositol; 1, 2, 3 = unknowns.

Fig. 4. Densitogram of pig gastric mucosa extract sample. Conditions as in Fig. 1. I = Myo-inositol; 1, 2, 3 = unknowns.

To test the stability of the dipping solution, a plate was cut into strips after development. The strips were dipped one after another at intervals of 1 h after mixing of the dipping solution. Each strip was measured 24 h after the dipping procedure.

RESULTS AND DISCUSSION

Myo-inositol (R_F value; 0.35) was separated from interfering components by the suggested solvent system (Figs. 1–4). Epi-inositol (R_F value 0.22) and myo-inositol 2-monophosphate (R_F value 0.85) were separated and detected by this method, too. Phytic acid (sodium salt) did not form a fluorescent spot.

The dipping solution is stable for at least 5 h after mixing (stored closed in darkness). The fluorescence intensity of the spots was observed to diminish (by about 15%) during this period.

The detection limit of myo-inositol was *ca.* 0.5 ng per spot. This limit was defined as the lowest concentration of myo-inositol that produced a signal about three times the average of the plate background noise (Fig. 5).

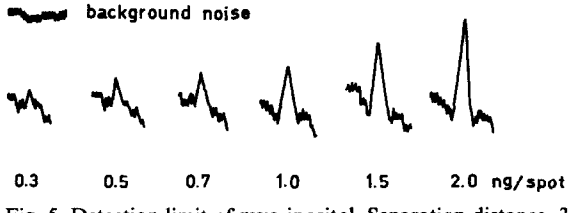

Fig. 5. Detection limit of myo-inositol. Separation distance, 3 cm; other conditions as in Fig. 1.

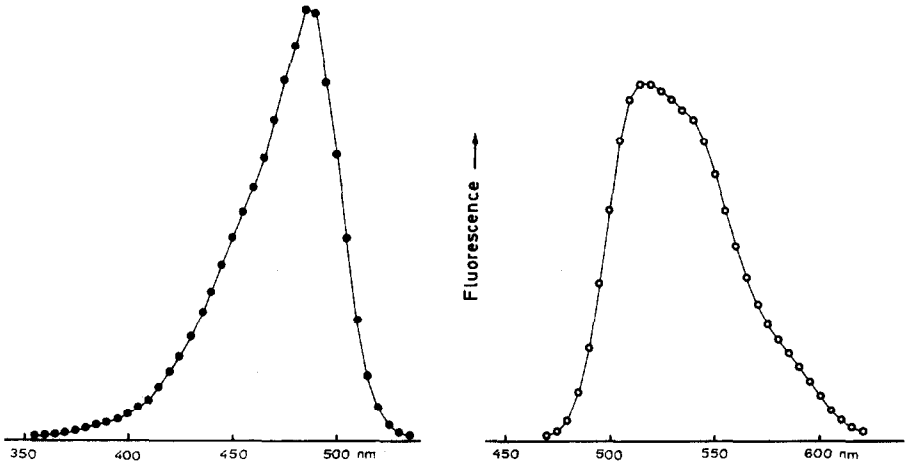

Fig. 6. *In situ* excitation (●) and emission (○) spectrum of myo-inositol after treatment with lead tetraacetate and sodium fluorescein.

The excitation and emission fluorescence spectra of myo-inositol are shown in Fig. 6. The spectra are identical with those of sodium fluorescein. The optimal conditions for the fluorescence measurements were determined from these spectra.

The calibration graph for myo-inositol shows a linear relationship between peak area and concentration from 1 to *ca.* 35 ng per spot, which allows one-point calibration ("100% method") to be used in this concentration range. The correlation coefficients of the calibration graphs were 0.992–0.999 (Fig. 7).

The results were calculated from the calibration line according to the least-squares method, using the data-pair technique[25]. To test the accuracy of the method, different batches of pharmaceutical preparations and organic extracts were analysed. The coefficients of variation were determined from five pairs of samples chromatographed on the same plate, and varied between 1.3 and 2.3% for the pharmaceuticals and 2.8 and 6.0% for the organic extracts. A sample of Prohepar dragées was developed in this way on each of five plates. The mean value from each plate was used for the calculation of the coefficient of variation.

A recovery test was carried out in the concentration ranges expected for myo-inositol in the organic extracts. The addition of known amounts of myo-inositol to the samples of the organic extracts gave recoveries from 97 to 108%.

Typical results for the determinations are given in Tables I and II. The results for the pharmaceutical preparations were well within the tolerance limits. The high recovery of myo-inositol from the spiked hip extract sample may be due to a matrix effect. Standards of other naturally occuring inositols were not available; they could interfere in the analysis of the organic extracts.

The myo-inositol spots have a yellowish colour after fluorescence detection with an *in situ* absorption maximum at *ca.* 450 nm. The measurements can be made in the reflectance mode, but the detection limit of myo-inositol is several times higher than that for the fluorescence method.

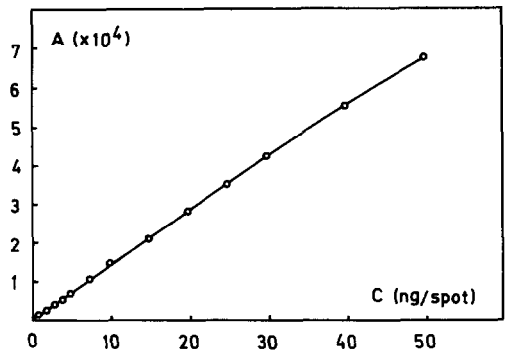

Fig. 7. Calibration graph for myo-inositol. Conditions as in Fig. 1. A = Peak area ($\times 10^4$ counts); C = concentration (ng per spot). $C = -0.125 + 7.30 \cdot 10^{-4} A$; correlation coefficient = 0.999.

TABLE I

DETERMINATIONS OF myo-INOSITOL IN PHARMACEUTICAL PREPARATIONS

Pharmaceutical preparation examined	*Myo-inositol found (%)**	*Coefficient of variation (%) (n = 5)*
Prohepar dragées	99.9	2.3; 2.7**
Proheparum-Gold dragées	96.8	2.4
Prohep tablets	96.0	1.3
Prohepar syrup	101.7	1.9

* As percentage of the label concentrations.
** On five different plates.

TABLE II

DETERMINATION OF myo-INOSITOL IN ORGANIC EXTRACTS

Organic extract examined	*Myo-inositol (%) (dry weight)*	*Coefficient of variation (%) (n = 5)*	*Recovery (%)*
Liver extract	0.435	6.0	103
Gastric extract	0.651	2.8	97
Gastric mucosa extract	0.586	3.7	102
Hip extract	0.535	4.1	108

CONCLUSION

The method described represents an improvement over other chromatographic methods. The *in situ* fluorimetric method is practicable, sensitive, accurate and possesses good reproducibility. Up to 44 samples can be run simultaneously on a 10

× 20 cm plate in a short time (*ca.* 10–20 min). The method is suitable for the routine analysis of free myo-inositol in pharmaceutical preparations and organic extracts.

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