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QUANTITATIVE ANALYSIS OF SOME BILE ACIDS BY DIFFERENTIAL SCANNING CALORIMETRY COMBINED WITH TLC

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ABSTRACT

An analytical method useful for the quantitative determination of some bile acids is proposed. The analysis is carried out in two steps. The first is based on the thin layer chromatographic (TLC) separation of the bile acids on alumina plates. The second is based on the application of differential scanning calorimetry (DSC), which permits the characterization and determination of the amount of compound contained in each spot. The DSC signal is proportional to the amount of sample present in the spot layer, while the various peaks and peak temperatures are used to identify the separated compound.

INTRODUCTION

In recent years there has been much interest in bile acids in internal medicine and in pharmacology. A reliable and simple method was needed for the separation and for the qualitative and quantitative determination of the bile acids in biological fluids. Several colorimetric methods have been proposed for the analysis of the bile acids, but all are non-specific. The enzymatic method described by Hurlock and Talalay [1,2] and applied by Iwata and Yamasaki [3] has been useful for the determination of the 3-hydroxy bile acids, particularly lithocholic (LA), cholic (CA), deoxycholic (DCA) and chenodeoxycholic (CDCA) acids.

The most suitable and practical method for the separation of the bile acids is thin layer chromatography (TLC), which is a technique widely applied in qualitative analysis. To obtain quantitative data, it is necessary to cut out the layer corresponding to the spot of the compound and then extract it with a suitable solvent. The quantitative determination is then carried out on the extracted compound using a suitable method. This technique has been applied to the bile acids by Bruusgard [4]. The method is not very accurate and the procedure is difficult and requires a long time.

We have applied differential scanning calorimetry (DSC) as a tool to

simplify and obtain quantitative data on the TLC spots [5]. Considering that aluminum oxide is the most common inert material used to prepare TLC plates, it is possible to carry out TLC on alumina plates, cut out the layer corresponding to the spot of interest, and to place it in the DSC sample capsule. The reference capsule is filled with an identical weight of the sample-free aluminum oxide obtained from the chromatographic plate. The DSC signal obtained due to the heat effect (thermal decomposition, melting, transition and/or some other) will be proportional to the quantity of sample present in the spot layer.

EXPERIMENTAL

Instrumentation

The DSC curves were obtained on a Du Pont Model 990 DSC cell and console. The sensitivity was 0.05 mcal sec^{-1} full-scale deflection on the first channel and 0.10 mcal sec^{-1} full-scale deflection on the second channel. A heating rate of 10°C min^{-1} was used. The furnace atmosphere consisted of dry oxygen at a flow rate of 100 ml min^{-1}. The peak areas were measured by means of a Linseis series 2000 integrator. The TLC plates were prepared by a Shandon apparatus, Mod. Unaplan.

Reagents

The cholanic acids were supplied by Nyegard, Oslo and Sigma Chemical, Saint Louis, MO, U.S.A. Aluminum oxide H and T for TLC were supplied by Merck, Darmstadt. All the other reagents were supplied by Merck, Darmstadt.

RESULTS

The alumina layers were prepared by mixing 30 g of adsorbent and 60 ml of water. The glass plates (20 × 5 and 20 × 20 cm) were covered with a layer of alumina (aluminum oxide H or T), the thickness of which ranged from 0.2 to 0.4 mm. After spreading, the plates were air-dried for 30 min, activated at 110°C for 45 min, and then stored in a desiccator.

A methanolic solution containing the compounds to be separated (25 μl) was placed on the TLC plate and dried in a warm air stream for about 10 min before elution was started. The methanolic solutions, each containing different quantities of each of the four acids, ranged in concentration so that in 25 μl there was a minimum of 25 μg and a maximum of 100 μg of each acid. All of the four acids were developed using a phosphomolybdic acid spray, 3.5% for TLC, from Merck, Darmstadt. After several runs, it was found that the best way of separating the four acids was by a double elution in the same direction on a plate with an alumina layer of 0.2 mm thickness. The two mixtures used were: (1) benzene—ethanol (95 : 5), and (2) butanol—propanol—ammonia (20 : 60 : 20). The results obtained were as follows

Bile acids	R_f obtained with	
	Mixture 1	Mixture 2
LA	0.80	0.98
DCA	0.37	0.97
CDCA	0	0.77
CA	0	0.38

The elutions were carried out on three identically treated plates, two of which were used as the reference and one for the sample. After the first elution with mixture 1, one reference plate was developed by phosphomolybdic acid. The sample plate and the second reference were then scored across the plate just below DCA spot, as seen on the developed reference plate. A second elution was then carried out using mixture 2 and, comparing it with the developed second reference, identified the positions of all the four acids. The position of the acid on the sample plate may also be identified by exposing the plate to iodine vapors [4]. In this case the spots appear brown. The iodine does not interfere with the DSC analysis. The spot areas of the alumina corresponding to each acid (1 cm^2) were then scraped off and placed in four capsules for DSC analysis.

Calibration curves

The calibration curves were obtained by weighing in two capsules exactly the same amount (18—22 mg) of the alumina used to prepare the TLC plates. Using a microsyringe, to one of the two capsules were added 25 μl of a methanolic acid solution, with a concentration range of 25—100 μl, of only one acid. In the second capsule 25 μl of pure methanol were added. The two capsules were then placed in an oven at 60° C to evaporate the methanol, and after this treatment analyzed by DSC. The DSC curves in Fig. 1 indicate an exothermic peak between 220 and 450° C for each acid. A calibration curve was then constructed by plotting the peak area vs. the acid weight. Many samples of each weight were prepared so that the data could be treated statistically. In this way the four calibration curves reported in Fig. 2 were obtained, with the equation and correlation coefficients for each summarized in Table 1.

To determine if the calibration curves so obtained were reproducible, the same sample masses were applied to the TLC plates and then eluted using the following procedure. Twenty-five μl of the acid solution used for the calibration curves were applied to three identical TLC alumina plates, two used as reference and the third as the sample plate. After elution with solutions 1 and 2, the second reference plate was developed, the positions of the spots identified and the corresponding areas on the sample plates removed and placed in a DSC capsule. Plots of the areas obtained vs. the mass of the acid sample gave a new series of calibration curves, which were reproduced within experimental error to those of the first series. A third series of data was ob-

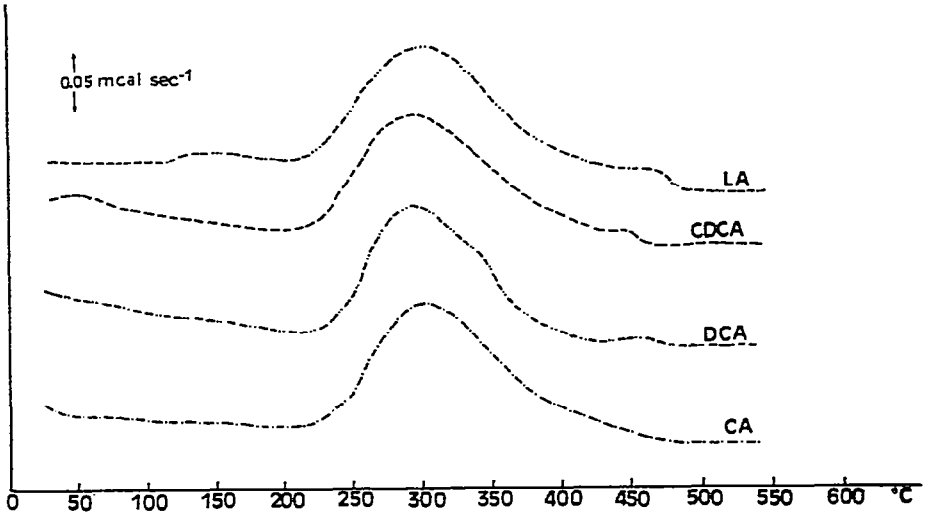

Fig. 1. DSC curves. Heating rate 10°C min^{-1}; dynamic oxygen atmosphere.

tained by applying 25 μl of mixtures of the four acids containing a minimum of 25 μg and a maximum of 100 μg of each acid to the TLC plates. As before, the areas corresponding to each acid spot on the sample were removed and placed in DSC capsules. Plots of the areas obtained vs. the mass

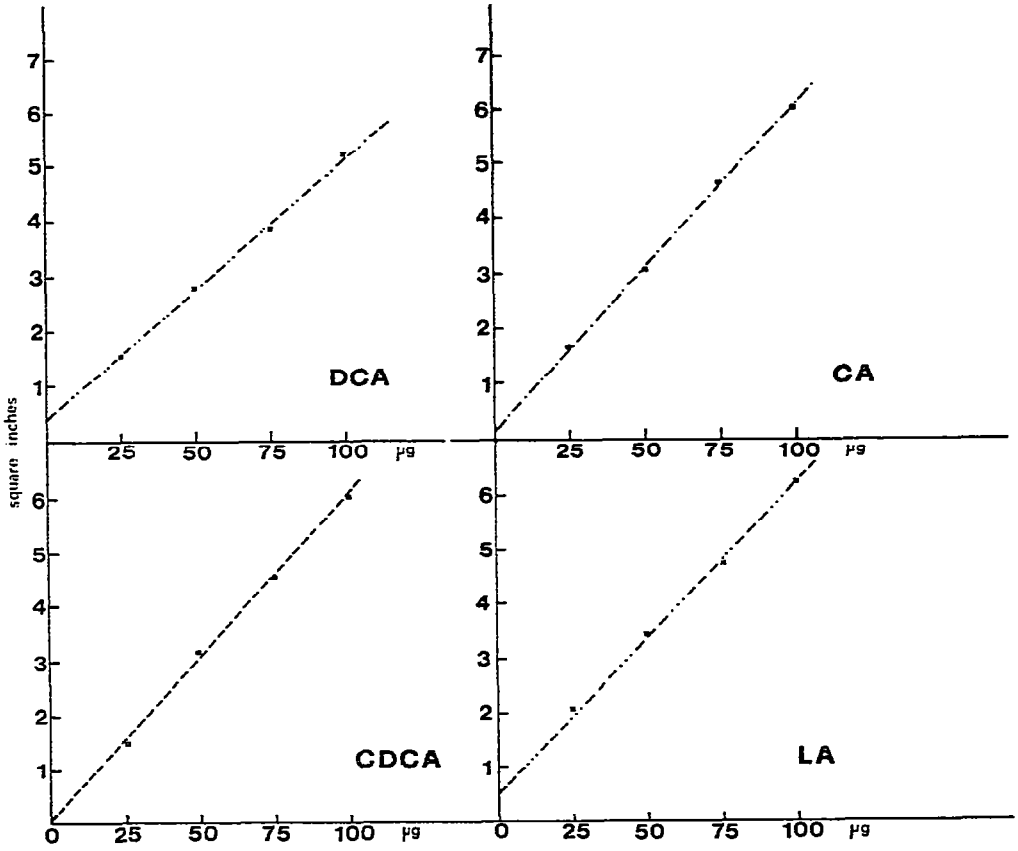

Fig. 2. Calibration curves.

TABLE 1

Equations and correlation coefficients of the four calibration curves

Acid	Straight line equation	Correlation coefficient r
Lithocholic	$y = 0.057x + 0.505$	0.94
Deoxycholic	$y = 0.047x + 0.430$	0.98
Chenodeoxycholic	$y = 0.060x + 0.083$	0.99
Cholic	$y = 0.059x + 0.161$	0.99

of the acid sample gave a third series of calibration curves that reproduced the other two series within experimental error.

DISCUSSION

The proposed analytical method is simple and requires only a short time to obtain quantitative data on the TLC plates. The DSC signal is directly proportional to the amount of substance present in the spot. The sensitivity is quite good and can be increased about 10 times using a more sophisticated DSC instrument, especially if it is coupled to a computer. The method is highly reproducible and the maximum error is about 2% under the worst conditions. With respect to the classic densitometric method, it appears that the densitometric method normally gives an error of about 5% minimum, if carried out in transmitted light, is not very reproducible, and depends upon the value of ϵ of the analyzed substance when colored or on the ϵ of the obtained colored compound when the substance is colorless, and needs to be developed with a proper reagent. As is known, when working in reflected light the measure is not quantitative [6].

The technique presented here is widely applicable to many separations that can be carried out on alumina TLC plates. It shows good possibilities of being applicable to other kinds of plate adsorbents because the DSC signals are differential and the only limiting parameter is the difference in mass between the amount of TLC layer present in the sample and reference capsules. Finally, the peak position on the DSC curve permits the qualitative identification of the bile acid present.

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