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QUANTITATIVE *IN SITU* THIN-LAYER CHROMATOGRAPHY OF QUINIDINE AND SALICYLIC ACID IN CAPILLARY BLOOD

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SUMMARY

A micro-method based on *in situ* thin-layer chromatography has been developed for the determination of quinidine and salicylic acid in 10-μl samples of serum or plasma. The sample is applied directly to the silica gel layer without extraction, the proteins being precipitated on the chromatogram origin by means of ethanol. The chromatogram can be evaluated quantitatively by fluorescence scanning. Different pre-coated plates were investigated.

INTRODUCTION

Methods for drug determinations in biological fluids usually need a more or less laborious extraction step prior to the quantitative measurement. Determination of the plasma level of a drug is often important in the clinic in order to achieve an optimum and safe dosage regimen; in pediatrics and in pharmacokinetic studies micro-methods using capillary blood are an advantage as venepuncture is avoided.

In a previous paper[1] a micro-method based on *in situ* thin-layer chromatography (TLC) was described for the assay of carbamazepine and metabolites in plasma. In this paper a micro-method for the determination of quinidine and salicylic acid without extraction is described. It is based on quantitative *in situ* TLC, only 10 μl of serum or plasma being needed for a single determination.

MATERIALS AND METHODS

Thin-layer chromatography

A layer of silica gel G 60 (Merck, Darmstadt, G.F.R.), 0.25 mm thick, was coated on 20 × 20 cm glass plates, which were air dried and activated during 80 min at 110° (10 plates). A horizontal groove was traced at a distance of 12 cm from the starting line for quinidine and at 10 cm for salicylic acid.

Reagents and reference standards

All chemicals used were Merck analytical grade.

Quinidine stock solution contained 1 g of quinidine in 1 litre of ethanol.

Aqueous dilutions corresponding to 25, 50 and 100 mg/l were prepared. From these aqueous dilutions reference serum (or plasma) standards corresponding to 1, 2 and 4 mg/l were prepared by adding 400 μl of the aqueous solutions to 10 ml of normal serum or plasma.

Salicylic acid stock solution contained 10 g/l of salicylic acid and was prepared by dissolving 1 g in 75.5 ml of hot 0.1 *M* sodium hydroxide and then diluting to 100 ml with water. Reference serum standards corresponding to 50, 100 and 200 mg/l were prepared as described for quinidine. The reference serum standards were stored for 6 months at —20° in small glass tubes (40 × 10/11 mm) sealed with polyethylene stoppers.

Instruments

A Vitatron TLD-100 densitometer fitted with UVB 366, 456 and 473-nm filters and a Hewlett-Packard electronic integrator 3370 B was used. Spectra were recorded on an Aminco Bowman spectrophotofluorometer fitted with an Aminco thin-film chromatograph scanner.

Procedure

For determining quinidine, 10 μl of ethanol were applied by means of a 500-μl Hamilton syringe with a repeating dispenser to the silica gel layer 1.5 cm from the lower edge of the plate; immediately afterwards, 10 μl of serum or plasma were applied on the ethanol spot. For application of serum or plasma, a 10-μl double-constriction pipette was used; the spots were applied 1.5 cm apart in the sequence standard 1, sample 1, sample 2, sample 3, sample 4, standard 2, sample 1, sample 2, sample 3, sample 4, standard 3. After application, the plates were dried for 10 min at 60° and then cooled to room temperature. The chromatogram was then developed in a saturated chamber (equilibration time at least 30 min), in the solvent system benzene, dioxan, ethanol, ammonia (25%) (50:40:5:5), for about 45 min. After development, the plate was dried for 10 min at 80° in a ventilating oven and, after cooling to room temperature, sprayed with a mixture of 8 ml of sulphuric acid (50%) and 2 ml of ethanol (the spraying must not be too vigorous).

The separated spots were quantitated by scanning the fluorescence on the Vitatron TLD 100 using a mercury lamp with excitation at 366 nm and emission at 456 nm. Calculation of the concentration in each sample was made from a calibration graph constructed for each plate.

Salicylic acid was assessed in the same way as for quinidine using the solvent benzene–diethyl ether–glacial acetic acid–methanol (60:30:9:0.5), and a development time of about 15 min. After development, the plates were dried for 5 min at 80° in a ventilating oven (no spraying is needed). Scanning was accomplished using an excitation UVB filter and emission at 473 nm.

RESULTS

In Fig. 1, the excitation and emission spectra for quinidine on silica gel G are shown. Fig. 2 shows a calibration curve obtained by *in situ* scanning of a chromatogram after direct application of 10 μl of serum containing different amounts of

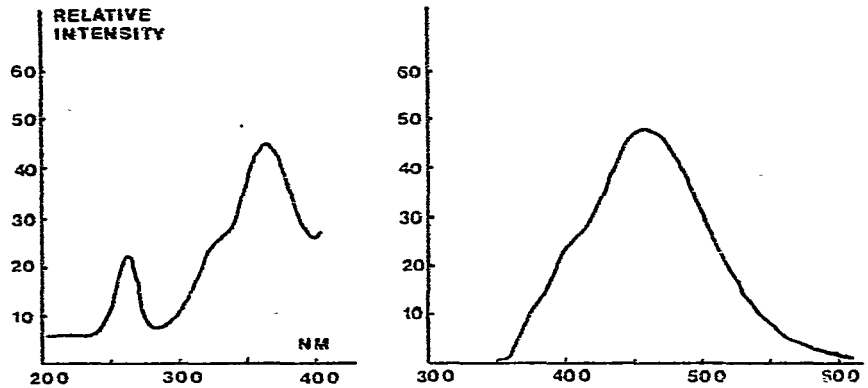

Fig. 1. Excitation and emission spectra for quinidine on silica gel G obtained on an Aminco Bowman spectrophotofluorometer fitted with Aminco thin-film chromatograph scanner. Emission spectrum obtained by excitation at 367 nm and excitation spectrum obtained with emission at 456 nm.

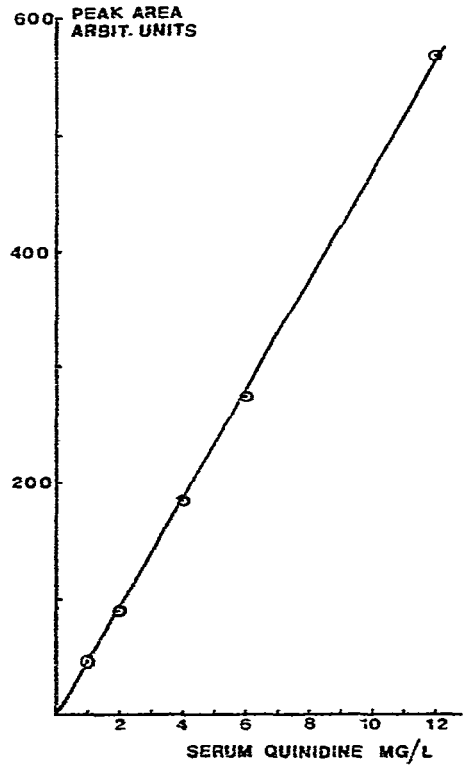

Fig. 2. Quinidine calibration curve obtained by *in situ* scanning of a chromatogram after direct application of 10 μl of serum.

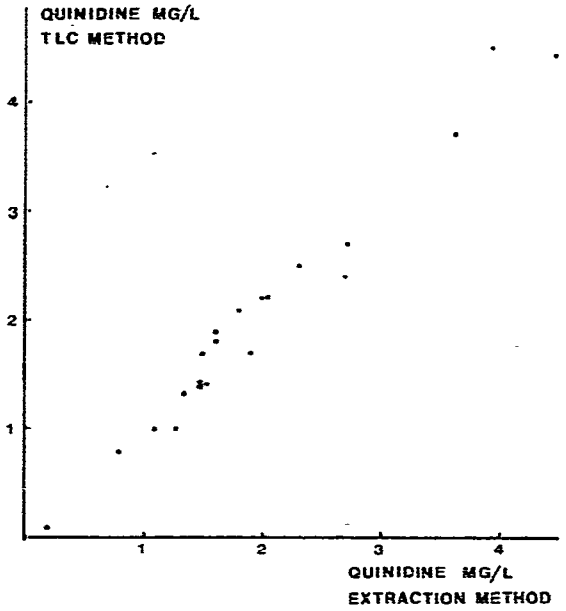

Fig. 3. Serum quinidine in 23 patients. Correlation of results between the TLC method involving direct application of 10 μl of serum and the double-extraction method of Cramér and Isakson[2] using 500 μl of serum.

quinidine. The calibration curve is linear up to 12 mg/l concentration; therapeutic concentrations are usually between 1 and 4 mg/l.

The correlation between the TLC method involving direct application of 10 μl of serum and the double-extraction method of Cramér and Isakson[2] using 500 μl of

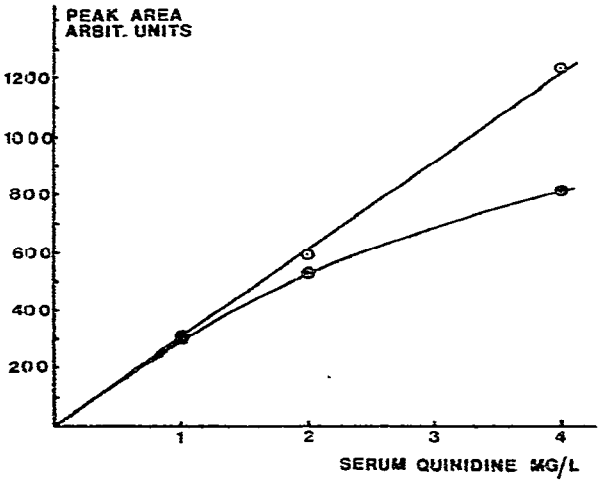

Fig. 4. Quinidine calibration curves obtained by *in situ* TLC scanning after direct application of 10 μl of serum on different silica gel layers. ●, Merck silica gel G 60; pre-coated plate; ⊙, laboratory prepared plate using Merck silica gel G.

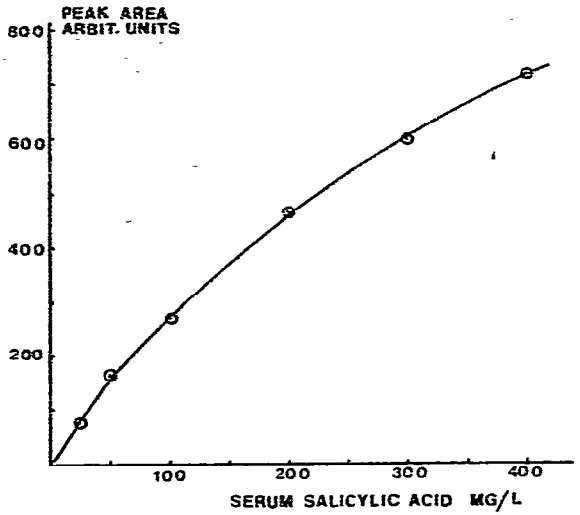

Fig. 5. Salicylic acid calibration curve obtained by *in situ* scanning of a chromatogram after direct application of 10 μl of serum.

serum can be seen in Fig. 3. The standard deviation calculated from the difference between duplicate determinations was $\pm$ 0.057 mg/l for the TLC method and $\pm$ 0.023 mg/l for the extraction method. The correlation coefficient was 0.983 ($n = 23$).

The use of pre-coated glass plates for direct application of serum was investigated for three different products, Merck silica gel G 60, Riedel de Häen Fertigplatten SI and Woelm silica gel. The quinidine calibration curve on Merck silica gel G 60 pre-coated plates shows marked curvature, even at 4 mg/l concentration (Fig. 4). The other two products exhibited calibration curves similar to those found by using silica gel plates prepared in the laboratory. The calibration curve for salicylic acid obtained by *in situ* scanning of a chromatogram on laboratory prepared silica gel plates with direct application of 10 μl of serum is shown in Fig. 5; the line is slightly curved. The precision of the salicylic acid method has been studied by means of replicate analyses of a patient's serum on different plates, and the mean value obtained was 111 $\pm$ 5.0 mg/l ($n = 18$). The calibration curve obtained by using pre-coated plates (Merck, Riedel de Häen and Woelm) were too curved to yield reproducible quanti-

TABLE I

COMPARISON OF SALICYLIC ACID SERUM LEVELS IN PATIENTS AS DETERMINED BY THE TLC MICRO-METHOD AND THE METHOD OF TRINDER[3]

TLC method (mg/l)	*Trinder procedure (mg/l)*	*TLC method (mg/l)*	*Trinder procedure (mg/l)*
0	10	27	49
2	15	17	54
3	21	33	95
12	33	48	99
18	120	61	110

tative measurements. The TLC micro-method has been compared with the method of Trinder[3] for the determination of salicylic acid in serum taken from 10 different patients and the results obtained are shown in Table I.

DISCUSSION

Chromatographic separation by TLC after direct application of serum or plasma usually gives poor results, due to the proteins in the sample. In the above described TLC micro-method, this difficulty has been avoided by precipitating the proteins at the starting line by means of ethanol. It is essential that the application of serum takes place immediately following the application of ethanol. By using this procedure the precipitated proteins remain at the starting line during the development and a clear separation of other substances is obtained. The sequence of sample and standard application described decreases the error caused by variations in the thickness of the silica gel layer.

The drying of the plate at 60° after the application is important in order to obtain uniform R_F values. The spots should also be well aligned after development, as the scanning is performed at right angles to the development. For the same reason serum or plasma reference standards are used to establish the calibration curves as pure substances exhibit different R_F values when compared with the same substances applied in serum or plasma. By use of serum or plasma standards the problems of recovery are also avoided.

In the literature several methods for the determination of serum quinidine have been described[2,4–8]. Results obtained with different methods vary considerably, extraction methods giving considerably lower results than methods that do not include extraction[9]. Extraction prior to fluorometric assay enables quinidine to be separated from its polar metabolites and other interfering substances in serum. Härtel and Harjanne[9] and Kessler *et al.*[10] consider the double-extraction method of Cramér and Isakson[2] to be superior to other methods because of its greater specificity and accuracy. In the method developed here, chromatographic separation takes place prior to the fluorometric measurement, and the results obtained correlate well with those obtained by the method of Cramér and Isakson, as shown in Fig. 3.

The possibility of using pre-coated glass plates for direct application of serum or plasma depends on the product (as shown in Fig. 4). The difference between the calibration curves obtained from laboratory prepared plates and pre-coated plates probably being due to the difference in the particle size of the silica gel. The size of the silica gel particles used on Merck pre-coated plates ranges from 5 to 20 μm, with 10 μm as the predominant size. The size of the particles in the silica gel G used to prepare plates in the laboratory, ranges from 5 to 60 μm with a wide distribution. The diffusion of a spot is less marked on layers with small particles and a clearer separation is obtained. On the other hand, quenching of the fluorescence will occur at lower concentration, resulting in a more curved calibration curve. The solvent system itself also exerts some influence when pre-coated plates are used in the case of quinidine but not for salicylic acid.

Most of the methods for the determination of salicylic acid in biological fluids described in the literature are based on photometric or fluorometric measurement, the photometric methods being based on the purple colour given by salicylate ion

with iron(III) nitrate in weakly acidic solution[3,11,12]. Lange and Bell[13] have described a fluorometric determination of acetylsalicylic acid and salicylic acid in whole blood (100-μl samples) after extraction and paper chromatography and elution of the paper strips. The method of Trinder[3], which is rapid, is based on a single reagent that precipitates the proteins and simultaneously gives a colour with salicylates; it is stated that the blank values for normal serum are small.

The TLC micro-method described in this paper gave considerably lower results than the Trinder method for salicylic acid in serum taken from patients (Table I). However, these patients included children who may well have consumed other drugs. Owing to the chromatographic separation and subsequent fluorometric measurement the TLC method is more specific. The methods developed here offer several advantages: they are performed on an ultramicro scale, and no solvent extraction or conventional protein precipitation is necessary. Separation of the active component from interfering biological substances by TLC and subsequent fluorometric measurement gives a high degree of specificity.

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