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THIN-LAYER CHROMATOGRAPHY FOR THE QUANTITATIVE SEPARATION OF QUINIDINE AND QUINIDINE METABOLITES FROM BIOLOGICAL FLUIDS AND TISSUES*

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SUMMARY

A chromatographic method for the study of quinidine metabolism is described. Quinidine, dihydroquinidine and metabolites were separated by thin-layer chromatography on Kieselgel G with methanol–acetone (4:1) as the solvent system. The fluorescing bands were divided into four main fractions and extracted with ethanol–acetone (1:1) from the adsorbent layer of the plate. The fluorescence of each fraction was measured with and without addition of acetic acid and sulfuric acid. A standard curve was used to transform fluorescence into µg quinidine base. The recovery from the plates varied between 75 and 95 per cent. The results obtained by this method were highly reproducible.

Amyl alcohol–benzene (1:1) was found optimal for the extraction of quinidine and its derivates from alkalized serum, urine and tissues. Removal of quenching substances and concentration of the quinidine bodies was achieved by transfer to sulfuric acid and second extraction with amyl alcohol–benzene. For further concentration, partial evaporation of the extraction solvent in a nitrogen atmosphere and at a temperature below 37° was used.

In recent years several methods have been published for the qualitative separation of chinchona alkaloids by thin-layer chromatography[1–3]. This paper describes in detail a method for the quantitative separation of quinidine, dihydroquinidine and their metabolites and the application of the method to the study of quinidine metabolism.

MATERIALS AND METHODS

Thin-layer chromatography

Adsorbent. Kieselgel G nach STAHL (Merck), pH 7.

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Solvents. Methanol p.a., acetone p.a., ethanol abs.

Procedure. Glass plates were coated with an adsorbent layer to a standard thickness of 0.5 mm using a Desaga applicator. The plates were air dried and activated for 2 h at 110°. Equally spaced vertical grooves were traced through the adsorbent layer to divide it into 2.5 cm wide strips. A horizontal groove was traced at the level selected for the solvent front at the end of the development. Quinidine bodies in amyl alcohol or in a mixture of amyl alcohol–benzene were applied to the starting line of each strip about 1.5 cm from the lower edge of the plate. The amount applied to a single strip was usually 1 to 5 μg and was determined exactly by applying a certain amount of solvent with a known quinidine concentration, using a Hamilton microsyringe. The chromatoplates were developed by the ascending technique in a rectangular chamber with ground lid at a temperature of 4°. The solvent system consisted of methanol and acetone (4:1). The time required was 40–50 min. The plates were dried under a stream of hot air and the positions of the fluorescent spots determined under U.V. light. Samples of commercial quinidine and dihydroquinidine were run for comparison. The strips were divided into fractions according to Fig. 1 and the adsorbent layer of each fraction was transferred quantitatively into a glass tube. After addition of 15 ml of a mixture of ethanol–acetone (1:1) the tube was shaken several times during 15 min and centrifuged. Five ml of the clear supernatant was transferred to another tube and the fluorescence was determined with and without addition of 2 drops of glacial acetic acid and 2 drops of 20% sulfuric acid[4,5] in a Beckman Ratio Fluorometer (phosphor sleeve position "360", primary filter UG 11, secondary filter Wratten 2 A). The apparatus was standardized, using 0.1 *N* sulfuric acid as a blank and 0.1 *N* sulfuric acid containing 0.1 μg quinidine base/ml to adjust fluorescence intensity by 50 or 100 on the scale. A standard curve obtained with quinidine in ethanol–acetone was used to convert the fluorescence into μg of quinidine base. The slope of the curve was checked each time with a known amount of quinidine in ethanol–acetone.

Extraction of quinidine derivates from biological fluids and tissues

Solvents. Amyl alcohol p.a., benzene p.a.

Procedure. To one part of serum or urine two parts of 0.1 *N* NaOH and 15 parts of a mixture of amyl alcohol and benzene (1:1) were added. The mixture was shaken vigorously for 5 min in a glass stoppered tube to transfer the quinidine bodies to the organic phase. After centrifugation for 10 min at 3000 r.p.m. part of the clear supernatant was used for the determination of quinidine, and the greater part was treated further for chromatography. Five ml was transferred to another tube and an equal amount of 0.1 *N* sulfuric acid was added. By shaking the tube vigorously quinidine bodies were transferred to the sulfuric acid, whereas quenching substances remained in the organic phase. Quinidine concentration was determined by measuring the fluorescence of the sulfuric acid. The remaining clear supernatant was transferred to another tube and a smaller amount of sulfuric acid was added to it to obtain concentration of quinidine bodies. The ratio of organic solvent to sulfuric acid had to be kept less than 15:1 to achieve complete recovery. After shaking the tube the sulfuric acid was transferred to another tube, made alkaline with 1 *N* NaOH, and an amount of amyl alcohol–benzene was added to give a ratio of organic solvent to inorganic phase of 2:1 at least. The tube was shaken and centrifuged as described. The organic phase

was transferred to another tube, and concentrated further by evaporation of the benzene in a nitrogen atmosphere. A small amount of the remaining solvent was added with a Hamilton microsyringe to 5 ml of 0.1 *N* sulfuric acid and its quinidine concentration was checked by determining the fluorescence. An amount of solvent calculated to contain 3.0 µg of quinidine bodies was pipetted on the starting line of the chromatoplate, but any amount between 1 and 5 µg gave good separation of fractions on the plate. Since urine contained relatively high amounts of quinidine bodies the first amyl alcohol–benzene extract could be used for chromatography.

Heart, liver, skeletal muscle etc. was cleaned of blood with dry filter paper and a small piece of tissue was taken for further analysis. About 1 g was found suitable, 0.2 to 0.5 g in the case of heart muscle. The tissue and 5 ml of physiological saline were ground in a Ultra-Turrax homogenizer to a homogenous suspension. The glass tube was weighed both empty and with its contents before and after homogenization. The losses due to suspension adhering to the grinding rod varied from 1 to 4% and were taken into account for the calculation of quinidine concentration. One ml of 1 *N* NaOH was added to the suspension and 30 ml of the mixture of amyl alcohol–benzene. The tube was shaken vigorously for 5 min and its contents treated further as described for serum and urine extracts.

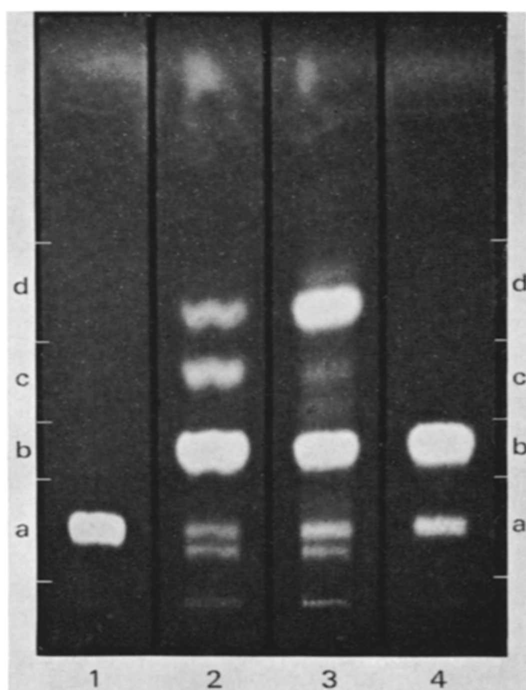


Fig. 1. Separation of quinidine, dihydroquinidine and metabolites by thin-layer chromatography. 1 = Dihydroquinidine, 2 = serum extract, 3 = urine extract, 4 = commercial quinidine contaminated with dihydroquinidine. Division into fractions which were extracted from the adsorbent layer is marked with *a*, *b*, *c*, and *d*. The plate was sprayed with sulfuric acid before U.V.-photography.

RESULTS

The separation of quinidine and quinidine derivates of serum and urine by thin-layer chromatography is shown in Fig. 1. For quantitative analysis the fluorescing

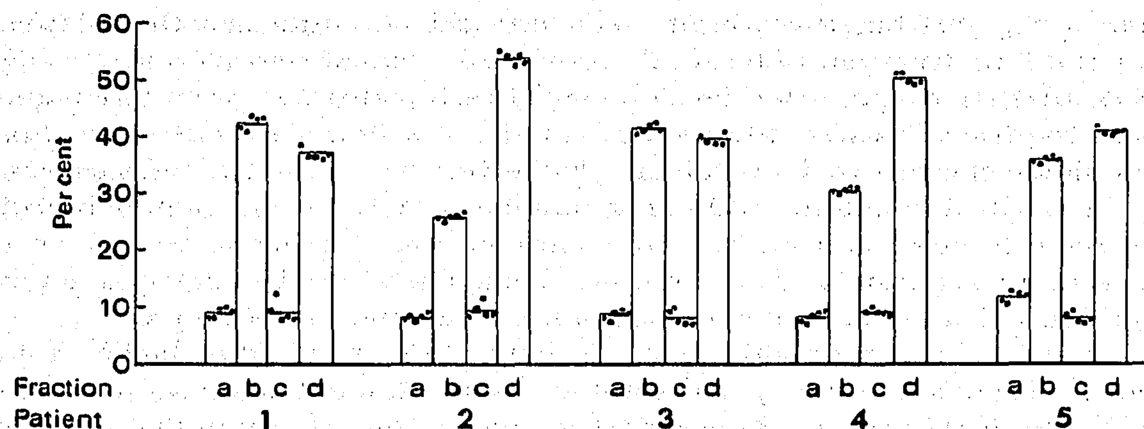
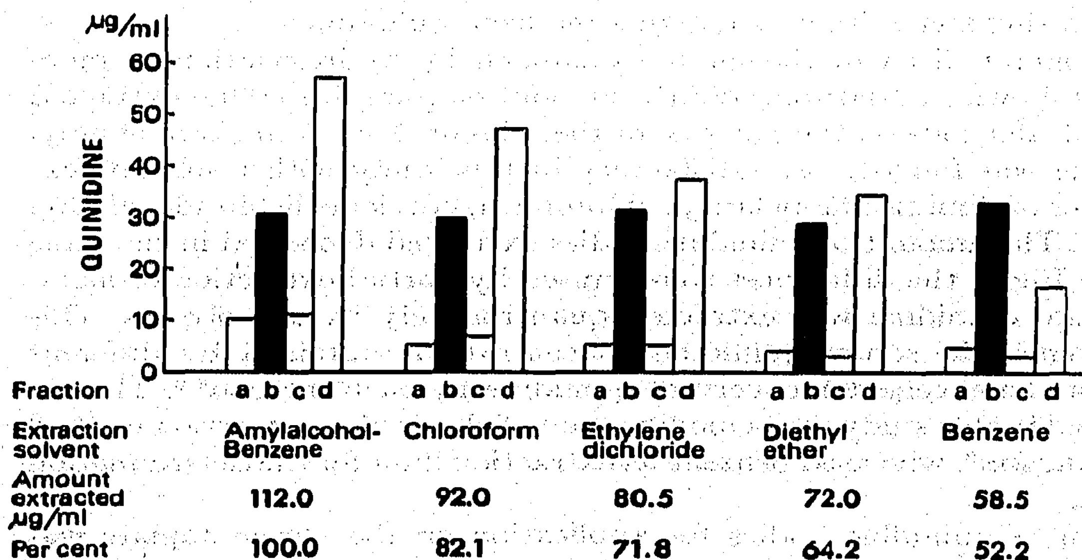


Fig. 2. Amyl alcohol–benzene extracts of five patients' urines separated into four main fractions by chromatography. The dots of each column represent values from five different plates.

bands of each strip were divided into four main fractions. Fraction *a* apparently contained dihydroquinidine and its metabolites; fraction *b* pure quinidine; fraction *c* the main quinidine metabolite circulating in blood and dihydroquinidine metabolites; and fraction *d* the main quinidine metabolite excreted into urine and a smaller amount of another quinidine metabolite. In addition, small amounts of several other metabolites were constantly found, and were located in the area of the fractions *a*, *c* or *d*.

In order to study the reproducibility of the chromatographic method, extracts of five different patients' urines were separated on five different chromatoplates each. The results are shown in Fig. 2. Fraction *c* showed the greatest relative variation, probably due to the difficulty of drawing a clear line between the fractions *b* and *c*, but even this variation was quite small. The total recovery from the plates varied



Extraction solvent	Amylalcohol-Benzene	Chloroform	Ethylene dichloride	Diethyl ether	Benzene
Amount extracted μg/ml	112.0	92.0	80.5	72.0	58.5
Per cent	100.0	82.1	71.8	64.2	52.2

Fig. 3. Effect of different solvents on the extraction of quinidine, dihydroquinidine, and metabolites from urine. Fraction *b* (black column) represents quinidine which was extracted quantitatively by all five solvents. The amount of metabolites extracted decreased with diminishing polarity of the solvent.

between 75 and 95%. Within these limits recovery did not influence the relative distribution of the four fractions. The total recovery decreased with the increasing age of the plates after coating or after development. Fresh plates were therefore made every week and the fractions were always extracted a few hours after the run. For commercial quinidine the recovery was about 5% better than for extracted samples. The recovery of purified quinidine, dihydroquinidine and the main metabolite of quinidine was found to be equal from the same chromatoplate. It made no difference if the samples were run separately or after mixing them. Therefore the relative amount of each fraction was calculated as a percentage of the total amount recovered.

The importance of the right solvent for the extraction of quinidine bodies from biological fluids is visualized in Fig. 3. The total amount extracted decreased with the diminishing polarity of the solvent. However, the amount of pure quinidine was the same independent of the solvent. The mixture of amyl alcohol–benzene was superior to all other solvents used because even more polar quinidine derivatives were extracted quantitatively by this system. The amount of fluorescing compounds in the residue after extraction with amyl alcohol–benzene was found to be negligible.

DISCUSSION

For the separation of chinchona alkaloids on an adsorbent layer of Kieselgel G, a solvent system containing diethylamine has been found to give optimum conditions by several authors[1–3]. The aim of the present study was to find a solvent system suitable for the study of quinidine metabolites during quinidine medication. As known, commercial quinidine preparations are usually contaminated with 10 to 15% of dihydroquinidine. A solvent system containing methanol and acetone (4:1) was found to give good and highly reproducible separation of quinidine, dihydroquinidine and their main metabolites. The addition of diethylamine was not necessary. Development of the plates at low temperature improved the quality of separation. The system is not suited for separating stereo-isomers like quinine from quinidine.

Quinidine is metabolized in the human organism by hydroxylation to more polar derivates[6]. As shown by BRODIE *et al.*[7] the amount of quinidine bodies extracted from biological fluids depends on the polarity of the solvent used. A mixture of amyl alcohol and benzene was found most satisfactory in this study. Other solvents examined were in order of diminishing polarity: chloroform, ethylene dichloride, diethyl ether, and benzene. The amount of quinidine bodies extracted decreased in the same order. As shown in Fig. 3 the differences were caused by partial extraction of metabolites, whereas pure quinidine was extracted quantitatively by all solvents. This explains the differences in serum quinidine concentrations obtained by different methods in spite of the excellent recovery of quinidine by each method[8,9]. The extraction method used in this study was a modification of the double extraction method of CRAMÉR AND ISAKSSON[9], who used benzene as extraction fluid for the determination of serum quinidine.

Concentration of quinidine bodies for application on the chromatoplate was achieved by varying the ratio organic–inorganic solvent and by partial evaporation of the second amyl alcohol–benzene extract. During evaporation a temperature below 37° and a nitrogen atmosphere was found necessary to avoid changes in the relative distribution of the quinidine fractions. A second transfer to sulfuric acid was obligatory

in the case of serum and tissue extracts to remove lipids and quenching substances. In the case of urine, the first amyl alcohol–benzene extract could be used for chromatography, as the quinidine concentration is relatively high. Transfer to sulfuric acid, to amyl alcohol–benzene, evaporation of the amyl alcohol–benzene mixture, or extraction from protein containing fluids did not change the relative distribution of quinidine and its metabolites.

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