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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROGUANIL, CYCLOGUANIL AND 4-CHLOROPHENYLBIGUANIDE USING HYDROPHOBIC PAIRING ION AND ITS APPLICATION TO SERUM ASSAY

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SUMMARY

A high-performance liquid chromatographic separation of proguanil, cycloguanil and 4-chlorophenylbiguanide is reported using a hydrophobic stationary phase and lauryl sulphate as pairing ion. It is suggested, on the basis of the behaviour of phenylbenzoate as an undissociated solute and the variation of retention with lauryl sulphate and sodium ion concentrations that the mechanism of separation is one of ion exchange. The biguanides can be detected in serum at concentrations in the region of 60 ng ml^{-1} and preliminary results are presented to show the variation of proguanil in serum over a 24-h period following ingestion of 200 mg orally.

INTRODUCTION

Proguanil hydrochloride (Paludrine, ICI) is widely used as a prophylactic agent against malaria. It is usually administered once daily as a 100-mg tablet although a 200-mg daily dose is recommended for parts of West Africa.

Proguanil is reported to be partially excreted unchanged from the body (40—60%) and partially metabolised to give cycloguanil as the main metabolite [1]. This metabolite has apparent antimalarial activity and it, rather than the parent drug, is thought to possess the prophylactic activity [2]. Subsequent metabolism results in the formation of another metabolite — 4-chlorophenylbiguanide [1].

Proguanil occasionally fails to provide a prophylactic effect despite strict adherence to the recommended dosage regime. This lack of prophylaxis has been attributed to acquired resistance by the plasmodia responsible [3]. In recent years apparent lack of activity or apparent toxicity of several drugs in humans has been well correlated with their pharmacokinetic parameters. The long-term

use of several drugs has been shown to result in the alteration of these parameters especially those relating to metabolism [4]. Since proguanil is administered on a daily basis over long periods of time and since metabolism apparently plays an important part in its activity there is a need for a detailed study of the pharmacokinetics of this drug and its metabolites.

The only pharmacokinetic data available [5] depend on relatively non-specific chemical assay. The method of high-performance liquid chromatography (HPLC) with its relative ease of sample preparation appears to offer the most suitable assay method for such a study. It is proposed to develop a chromatographic separation of these biguanides and to assess its suitability for serum assay of these compounds.

Since the advent of reversed-phase ion-pair chromatography [6—9] separations using this technique have been widely applied. Despite this, the mechanism of retention on hydrophobic phases using hydrophobic pairing ions is still under discussion [10—12] and relatively few data have been reported distinguishing between the ion-pairing concept and the alternative ion-exchange interpretation of retention [13—15]. It is proposed to examine the effect of lauryl sulphate and sodium ion concentrations on the separation of these compounds to obtain additional information on the mechanism of retention.

EXPERIMENTAL

Apparatus and materials

The liquid chromatograph used consisted of an Applied Chromatography System syringe pump linked to a Pye Unicam LC-3 ultraviolet detector. The chromatographic column (100 mm × 4.6 mm I.D.) was slurry packed at 200 bar with 5-μm Hypersil ODS (Shandon Southern, London, Great Britain). Syringe injection was used for the measurement of column capacity factors and for quantitative measurements a Rheodyne Model 7120 injection valve fitted with a 100-μl loop was used. The wavelength of measurement was optimised for proguanil at 247 nm and all measurements were made at ambient temperature. Acetonitrile was HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). All other chemicals used were obtained from BDH Chemicals (Poole, Great Britain). Proguanil, cycloguanil and 4-chlorophenylbiguanide were donated by ICI (Macclesfield, Great Britain).

Chromatography

A solution (20 μg ml^{-1}) in acetonitrile of the three biguanides was prepared containing phenylbenzoate (40 μg ml^{-1}) as a non-ionisable reference compound. The retention times of the four compounds were measured as a function of anion concentration over the range 0—0.909% w/v by adding volumes of 10% w/v sodium lauryl sulphate (SLS) solution in buffer together with an equal volume of acetonitrile to an eluent of acetonitrile—water (50:50) containing 0.175 *M* phosphoric acid and 0.0125 *M* sodium dihydrogen phosphate as a buffer. The pH of the eluent was 1.5. The retention times were also measured as a function of sodium counter ion concentration using a fixed SLS concentration of 0.7% w/v adjusted to pH 1.5 with sulphuric acid to which sodium sulphate solution

(1.33 *M*) was added together with an equal volume of acetonitrile to provide a range of sodium ion concentration from 0.07 to 0.313 *M*.

Calibration procedures for biguanides

Calibration curves for the three biguanides were run both in water and in bovine serum by spiking to provide concentrations in the range 80—450 ng ml^{-1} for each compound. The solutions in water were injected directly. The spiked serum was deproteinated by adding 0.25 ml of perchloric acid (60% w/v) to 1 ml of serum, shaking and centrifuging for 15 min. The clear supernatant was then injected. For quantitative measurements the eluent strength was modified to acetonitrile—water (75:25) and the SLS concentration to 2.5% w/v.

Determination of partition coefficients and dissociation constants

The partition coefficients for the three biguanides and phenylbenzoate were determined using an octanol—water system by measuring the absorbance of each compound at an appropriate wavelength in acid aqueous (adjusted to pH 1.5 with sulphuric acid) solution before and after equilibrating with a suitable volume of 1-octanol. The dissociation constants of the biguanides were determined by a spectrophotometric method [16]. A plot of pH against change in absorbance over that in the fully protonated form enabled the pK_a to be determined from the point of inflexion.

RESULTS AND DISCUSSION

Fig. 1 shows the separations obtained of the three biguanides and phenylbenzoate in the presence and absence of surfactant. It was noted that the order of elution of cycloguanil and 4-chlorophenylbiguanide was reversed on addition of surfactant.

Surfactant concentration

The results of increasing SLS concentration are shown in Fig. 2. The capacity ratios for the three biguanides are seen to increase linearly with increase in SLS concentration (R^2>0.956). The slopes of the lines are markedly different and are in the same order as the octanol/water partition coefficients, measured in acid solutions, for cycloguanil, 4-chlorophenylbiguanide and proguanil as 0.05, 0.11 and 0.33 respectively. The primary pK_a values of the bases at 20°C were determined as 4.2, 1.9 and 3.0 respectively and it is assumed that in the eluent used these compounds are appreciably protonated. The behaviour of the uncharged phenylbenzoate, showing decreased capacity ratio with increasing SLS concentration indicates that the surfactant is interacting directly with the stationary phase rather than in solution with the mobile phase.

Fig. 3 shows the effect of surfactant concentration on the reduced plate height (h). Data were obtained from chromatograms which allowed reliable measurement of column efficiency, i.e. where no overlap of peaks occurred. For proguanil and 4-chlorophenylbiguanide h is initially very large and tends, as the surfactant concentration increases, to the low constant value shown for phenylbenzoate which is independent of surfactant concentration. It would

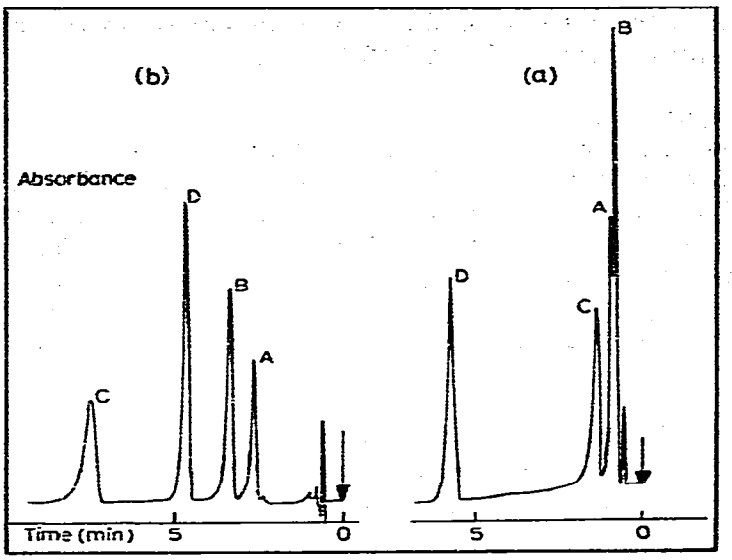

Fig. 1. Representative chromatograms to show the effect of sodium lauryl sulphate on retention. (a) No SLS; (b) 0.654% w/v SLS. Conditions: eluent acetonitrile—water (50:50) + 0.17 *M* H_3PO_4 + 0.0125 *M* NaH_2PO_4. Flow-rate, 1.5 ml min^{-1}; 10-μl syringe injection. Solute concentrations approximately 20 μg ml^{-1}. Full scale absorbance 0.08. Peaks: A, cycloguanil; B, 4-chlorophenylbiguanide; C, proguanil; D, phenylbenzoate.

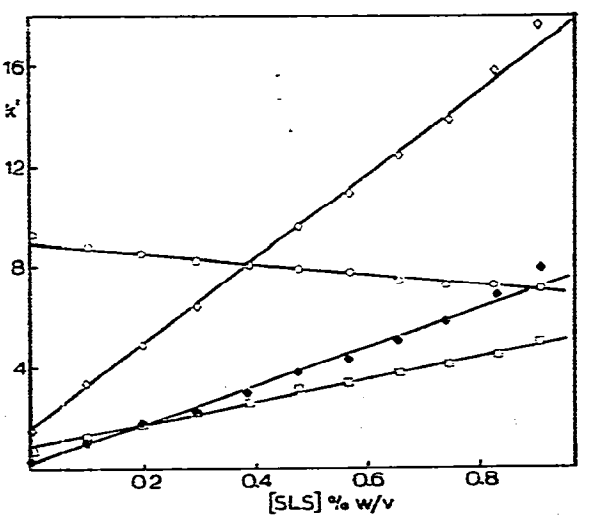

Fig. 2. Effect of surfactant concentration on capacity ratio (k') for cycloguanil (▫), 4-chlorophenylbiguanide (♦), proguanil (◇) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

appear from these results that high concentrations of surfactant are advantageous in that they render the putative ion-exchange mechanism of comparable efficiency to that obtained for uncharged solute species on reversed-phase systems. This is interpreted as evidence of an ion-exchange mechanism where

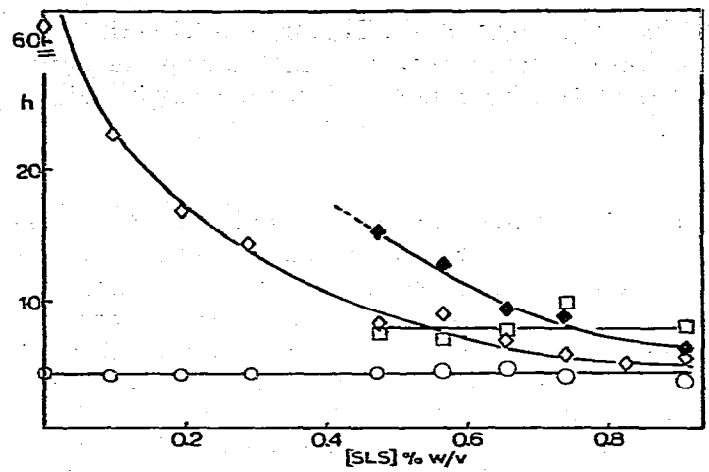

Fig. 3. Variation of reduced plate height (h) with surfactant concentration for cycloguanil (□), 4-chlorophenylbiguanide (◆), proguanil (◇) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

an effectively low capacity resin is being formed at low surfactant concentration producing minimal retention with consequent peak broadening due to overloading of the ionic sites available. In the present results there appears to be no evidence of the complex dependence of h on surfactant concentration demonstrated for tryptophan in a similar chromatographic system [17]. The present results are similar to those obtained for sodium cromoglycate with alkylbenzyldimethylammonium chlorides [12]. These results favour an equilibrium of the ion-exchange type represented by

$$(P^-Na^+)_{org} + BH^+_{aq} \rightleftharpoons (P^-BH^+)_{org} + Na^+_{aq}$$

where P^- refers to the surfactant anion and BH^+ to the protonated base. It has been shown [18] that in such a system the distribution ratio of the drug between the organic and aqueous phases and thus the chromatographic capacity factor k' is given by an expression of the type

$$k' = K_{IE} \frac{[P^-Na^+]_{org}}{[Na^+]_{aq}} \quad (1)$$

K_{IE} being equilibrium constant for the ion-exchange reaction shown.

If the surfactant partitioned into the stationary phase is in equilibrium with that in the aqueous phase as is likely in the case of a surfactant where micellisation forms an alternative to the removal of surfactant from the aqueous phase then

$$k = \frac{[P^-Na^+]_{org}}{[P^-Na^+]_{aq}}$$

where k is an overall distribution coefficient for SLS between the aqueous and the organic phases and

$$k' = K_{IE}k \frac{[P^-Na^+]_{aq}}{[Na^+]_{aq}} \quad (2)$$

which is the situation observed in the present work. This may not be the case where a pairing ion is unable to form micelles in which case continuous partitioning of the surfactant into the stationary phase will occur and $[P^-Na^+]_{org}$ will be independent of $[P^-Na^+]_{aq}$.

Counter ion concentration

Eqn. 2 shows that at fixed $(P^-Na^+)_{aq}$ concentration, k' should vary as the reciprocal of $[Na^+]$. The results of varying the sodium ion concentration are shown in Fig. 4. The phenylbenzoate is seen to be unaffected by sodium ion while the column capacity factors for the protonated species are linearly related to $1/[Na^+]$. This result further supports the ion-exchange interpretation of retention and indicates competition for anionic sites between sodium and protonated base. It can also be observed in Fig. 4 that in the presence of a large concentration of added sodium ion the retention of all bases is greatly reduced and the elution order of cycloguanil and 4-chlorophenylbiguanide reversed to that found in low surfactant concentrations. In these experiments no significant change in h as a function of sodium ion concentration was observed.

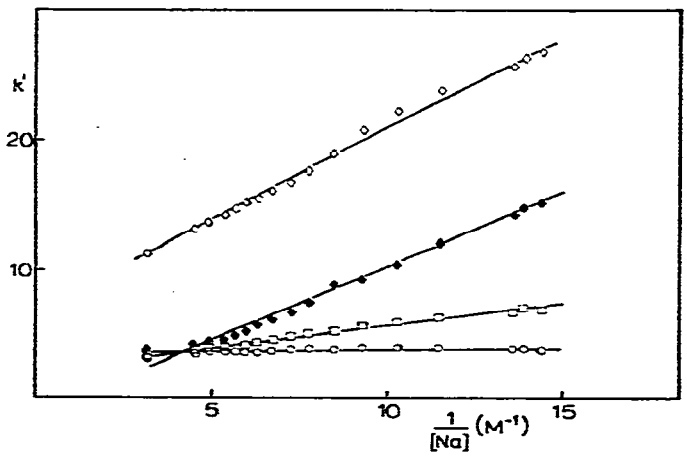

Fig. 4. Variation of the capacity ratio (k') with the reciprocal of sodium ion concentration for cycloguanil (□), 4-chlorophenylbiguanide (●), proguanil (○) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

Serum determinations

The blank and spiked bovine serum after protein precipitation and injection produced chromatograms as shown in Fig. 5. The three bases are well resolved and, with the exception of cycloguanil, well removed from endogenous plasma constituents. The calibration curves of peak height against concentration (C) were linear and the regression lines represented as

Cycloguanil peak height (mm) = $0.268C$ (ng ml^{-1}) + 1.115 ($R^2 = 0.990$, S.D. = $5 \cdot 10^{-3}$)

4-Chlorophenylbiguanide peak height (mm) = $0.185C$ (ng ml^{-1}) + 0.53 ($R^2 = 0.990$, S.D. = $6 \cdot 10^{-3}$)

Proguanil peak height (mm) = $0.129C$ (ng ml^{-1}) + 0.294 ($R^2 = 0.996$, S.D. = $5 \cdot 10^{-3}$)

at a full scale absorbance of 0.01.

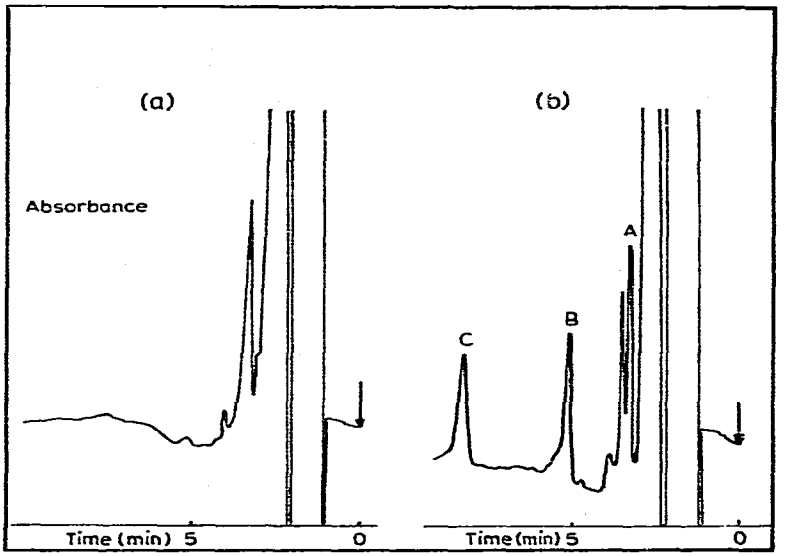

Fig. 5. Representative chromatograms to show the separation of biguanides in serum. (a) Blank bovine serum sample; (b) bovine serum sample containing 150 ng ml^{-1} of each biguanide. Peaks: A, cycloguanil; B, 4-chlorophenylbiguanide; C, proguanil. Conditions: eluent, acetonitrile—water (75:25) + 2.5% w/v SLS + 0.17 *M* H_3PO_4 + 0.0125 *M* NaH_2PO_4. Full scale absorbance 0.005, 100-μl valve and loop injection.

To estimate losses during the precipitation of protein comparable concentrations in water were injected directly. From the relative slopes of the lines obtained in water and in serum it was estimated that losses in the region of 10% were incurred during the deproteinating step.

The limit of detection for the three compounds was estimated to be in the region of 60 ng ml^{-1}. Attempts to improve this detection limit by selective extraction and concentration before injection were unsuccessful.

In vivo measurements

Since no information was available in the literature concerning human serum levels of proguanil following the ingestion of a prophylactic dose a trial run was made on a human volunteer. At varying times after ingestion of 200 mg proguanil blood samples were taken by venipuncture. Five ml of each sample were centrifuged after clotting and serum obtained. This was assayed by the above method. Specimen chromatograms are shown in Fig. 6. In these samples only proguanil could be detected and no clear evidence of either of the two accepted metabolites was found. The variation of serum proguanil with time is shown in Table I. This assay method was also applied to whole blood after lysing by repeated freezing and an increased proguanil concentration of 340 ng ml^{-1} was determined for the 1.5-h sample compared with 220 ng ml^{-1} for the serum alone.

In view of the paucacity of information on the pharmacokinetics of proguanil it is intended to apply the method described in a complete study. It appears that refinement of the method will be required if the presumably lower levels of the accepted metabolites are to be monitored.

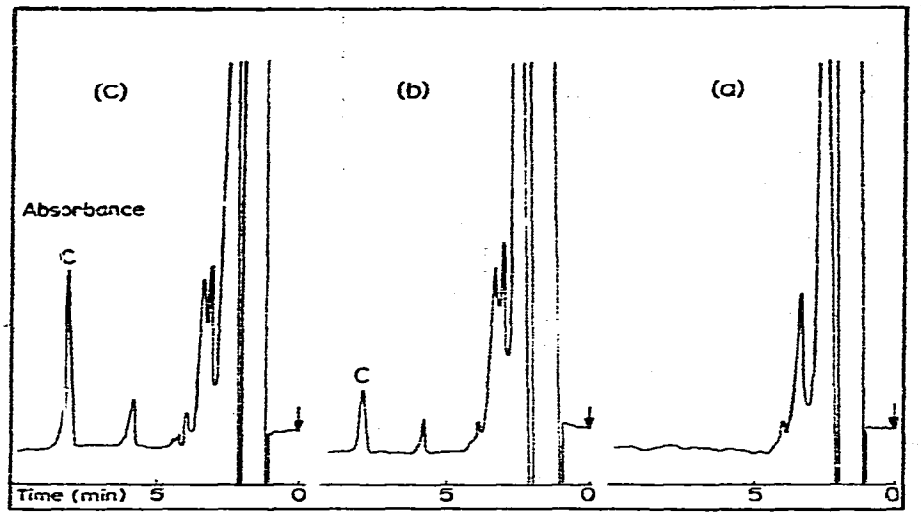

Fig. 6. Representative chromatograms of volunteer serum samples assayed after ingestion of 200 mg proguanil. (a) Blank serum sample; (b) serum after 0.5 h; (c) serum after 2.5 h. Chromatographic conditions as in Fig. 5. Peak C, proguanil.

TABLE I

RESULTS OF HUMAN SERUM DETERMINATION OF PROGUANIL AT VARIOUS TIMES AFTER INGESTION OF 200 mg PROGUANIL

Time (h)	0	0.5	1.5	2.5	3.5	24
Proguanil serum concentration (ng ml^{-1})	0	95	220	280	275	70

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