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USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE MEASUREMENT OF *IN VITRO* ACETYLATION IN MAN

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SUMMARY

Liquid chromatographic methods were developed for the study of the *in vitro* acetylation of the sulphonamide drug sulphamethazine and a series of aniline derivatives. The sensitivity of the methods have allowed data on the activity of the N-acetyltransferase enzyme(s) in man to be obtained. The use of bonded-phase columns with a series of organic mobile phases has been compared with reversed-phase separation of the acetanilide derivatives, produced in the acetylation reaction.

INTRODUCTION

Many drugs containing an aromatic amine or hydrazine group are metabolised in man by acetylation of the amino function. Such reactions are catalysed by a group of enzymes known as N-acetyltransferases, which are present in various tissues, their main site of action being the liver[1]. The activity of these enzymes towards certain amines is determined by genetic factors. Thus people can be classed as fast or slow acetylators of drugs, such as dapsone, isoniazid or hydralazine, depending on the rate at which they produce the corresponding acetylated metabolites. Knowledge of an individual's acetylator phenotype may be clinically important, since toxic side effects have been reported in fast and slow acetylators whose treatment was not related to their acetylator status[2]. The effect of the enzymes involved in these reactions can be measured by an analysis of the free and acetylated amine, following a standard dose of a test drug. The determination of acetylator phenotype is most frequently made with the drug sulphamethazine. The plasma or urine concentration of the drug at a particular interval following a standard dose reflects the subject's acetylator status[3].

The most widely used method for this type of analysis has been the Bratton–Marshall colorimetric assay[4]. This involves diazotisation of the free amine and coupling to N-naphthylethylenediamine. Hydrolysis of the amide to the amine, followed by a second diazotisation and coupling reaction, then gives the total amine value, from which the percentage acetylation can be calculated. The amount of acetylated product is thus calculated indirectly. Recently, liquid chromatographic methods have been developed for acetylator phenotyping[5]. We report here liquid chromatographic

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methods which we have developed for studying the acetylation of sulphamethazine and aromatic amines, such as aniline and phenetidine in blood.

EXPERIMENTAL

Materials

Sulphamethazine, acetic anhydride, acetanilide, aniline, and substituted acetanilides were obtained from Aldrich (Gillingham, U.K.). Potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from BDH (Poole, U.K.). HPLC-grade acetonitrile was obtained from Rathburn (U.K.). Ethyl acetate (Proanalysis grade), chloroform, dichloromethane, tetrahydrofuran and propan-2-ol were purchased from May and Baker (Dagenham, U.K.). Acetylsulphamethazine was synthesised from sulphamethazine by heating with acetic anhydride and was recrystallised from aqueous ethanol (m.p. 254–255°C). Its structure was confirmed by mass spectrometry and its purity was 100% by high-performance liquid chromatographic (HPLC) analysis. Ethyl acetanilide was prepared in a similar manner from ethylaniline, and its purity was checked by HPLC and mass spectrometry.

Instrumentation

HPLC analysis of sulphamethazine and acetylsulphamethazine. An Altex 100A pump (Beckman-RIIC, High Wycombe, U.K.) was used controlled by an Altex 420 program controller. A mobile phase of acetonitrile–33.3 m*M* phosphate buffer (pH 7.4) (15:85) was pumped at 1 ml min^{-1}. Samples were introduced through an Altex 210 valve (20 μl loop). The column was a 250 mm × 4.6 mm I.D. Altex Ultrasphere ODS (5 μm). A Pye Unicam LC3 UV detector (Pye Unicam, Cambridge, U.K.) was used at a wavelength of 240 nm. The detector signal was monitored by a Hewlett-Packard 3388A integrator (Hewlett-Packard, Altrincham, U.K.). When sulphapyridine was used as an internal standard, a Partisil 5 (5 μm) ODS column was used with a 10:90 mixture of the same mobile phases. With these systems the detection limit for the free and acetylated sulphamethazine was approximately 50 ng ml^{-1} at a signal-to-noise ratio of 2.

HPLC analysis of acetanilide and related amines and amides. A Gilson 302 reciprocating pump (Gilson Medical Electronics, Villiers-le-Bel, France) was used with a Rheodyne injection valve (20 μl, Rheodyne Cotati, CA, U.S.A.). A Pye Unicam LC3 detector was used at 235 nm. The detector signal was fed to a Rikadenki chart recorder (10 mV f.s.d.) (Rikadenki Misui, U.K.) and a LDC CI-10 integrator (Laboratory Data Control, U.K.). The column was a 250 mm × 4.6 mm I.D. Altex Ultrasphere ODS (5 μm). For reversed-phase chromatography a mobile phase of methanol–66.6 m*M* phosphate buffer (pH 7.4) (40:60) was used at a flow-rate of 1 ml min^{-1}. This flow-rate was also used for the normal-phase system.

Sulphamethazine experiments

All subjects were healthy male and female volunteers. Blood was taken by venepuncture at 9.0 a.m. into lithium heparin tubes before its immediate use. The incubation protocols were as follows: 0.5 ml of heparinised whole blood and 0.5 ml of a sulphamethazine solution in 33.3 m*M* phosphate buffer (pH 7.4) were mixed in 10 ml glass blood bottles to produce final concentrations of sulphamethazine in the

range of 18 μ*M* to 1.44 m*M*. The tubes were incubated for 24 h at 37°C with rotary mixing. Samples were then extracted into 5 ml ethyl acetate by 20 s vortex mixing. After an equilibration time of 90 min, a 4 ml aliquot of the organic layer was removed and evaporated to dryness at room temperature under a stream of nitrogen. The dried extract was then redissolved in the HPLC mobile phase and analysed. For construction of the calibration graphs sulphapyridine was dissolved in ethyl acetate to produce concentrations in the range of 5 μ*M* to 0.4 m*M*. These solutions were then used in place of ethyl acetate in the extraction procedure.

Initial studies on the extent of acetylation of sulphamethazine in blood indicated that after 24 h the maximum percentage acetylation would be of the order of 10%. To calibrate the system, mixtures of the free and acetylated sulphamethazine were prepared in ratios corresponding to an acetylation of 0 to 10%. They were prepared in 33.3 m*M* phosphate buffer (pH 7.4). Appropriate mixtures of the pure synthetic standards were prepared for each of the three sulphamethazine incubation levels to be used (18 μ*M*, 180 μ*M* and 1.44 m*M*). After addition of a standard to whole blood, it was immediately extracted. The ratio of the acetylsulphamethazine/sulphamethazine peak height was then plotted against the value of the particular percentage acetylation mixture used. For standards extracted using sulphapyridine as the internal standard the known ratio acetylsulphamethazine:sulphamethazine in the standard solution was plotted against the ratio of the peak height (acetylsulphamethazine:sulphapyridine).

Aromatic amine experiments

A volume of 0.2 ml of heparinised whole blood and the amine (5–50 nmoles) in 0.2 ml 66.6 m*M* phosphate buffer (pH 7.4) were mixed in 10-ml glass blood bottles. After incubation at 37°C for the appropriate time, the reaction was stopped by the addition of 4 ml of diethyl ether. An internal standard of 4-fluoroacetanilide (10 nmoles) in 100 μl of phosphate buffer was then added. After shaking for 2 min, the tube was placed in dry ice to freeze the aqueous layer, the ether layer was decanted and then evaporated under nitrogen at room temperature. The residue was dissolved in 50 μl of the HPLC eluent prior to injection. The extraction efficiency for acetanilide from whole blood was found to be less than 70%, and thus a method using an internal standard was chosen. To calibrate the assay, a range of acetanilide standards (0–10 nmoles) was added to whole blood and immediately extracted as described. Calibration graphs were prepared using a standard linear regression model, implemented on an Apple II microcomputer[6].

RESULTS

The calibration lines for sulphamethazine and its acetyl derivative, obtained with or without internal standard, were linear ($r > 0.99$) over the acetylation range of interest. The correlation between the two methods was determined on fifteen samples and gave a correlation coefficient $r > 0.99$. The precision of the assay at a substrate level of 18 μ*M* when monitoring an acetylation of 1.1% was 13.1% ($n = 5$). At this level the amount of acetylated material injected is approximately 2 ng. The precision at the same substrate concentration with an acetylation of 10.0% was 6.7%. The corresponding values at substrate levels of 180 μ*M* and 1.44 m*M* were 5.9,

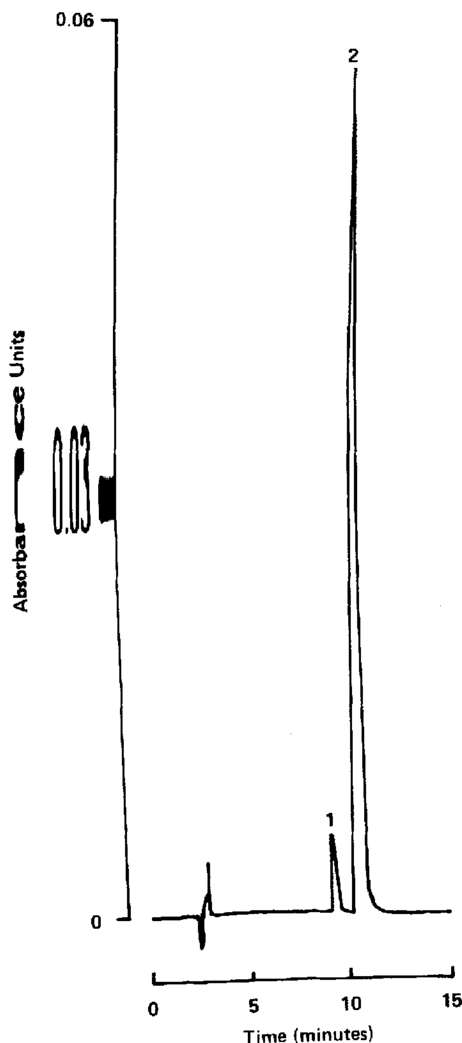

Fig. 1. HPLC tracing of acetylsulphamethazine (1) and sulphamethazine (2) following a 24-h incubation of 180 μM sulphamethazine. For HPLC conditions see text.

0.7, 2.9 and 0.4%, respectively. Although the recovery of sulphamethazine from blood was high ($>97\%$, $n = 15$), the corresponding figure for the acetylated material was $83.1 \pm 2.4\%$ ($n = 15$). We thus felt that the use of a calibration method was essential, especially where low levels of the acetylated drug would be produced.

The chromatogram shown in Fig. 1 is from an incubation of 180 μM of sulphamethazine over 24 h. The acetylated drug corresponds to approximately 7% of the substrate. Six substrate concentrations of sulphamethazine in the range 18 μM–1.44 mM were used to estimate the V_{max} and k_M of the blood N-acetyltransferase. The limited solubility of sulphamethazine in aqueous solution prevented study of higher substrate concentrations. An incubation time of 7 h was used for this experiment, since at this time the rate is still linear at all the concentrations studied. Estimates of V_{max}, the maximum catalytic rate and K_M, the substrate concentration which gives half the maximum velocity, were obtained from these data. These values were 74 picomoles min^{-1} ml^{-1} blood and 334 nanomoles ml^{-1}, respectively.

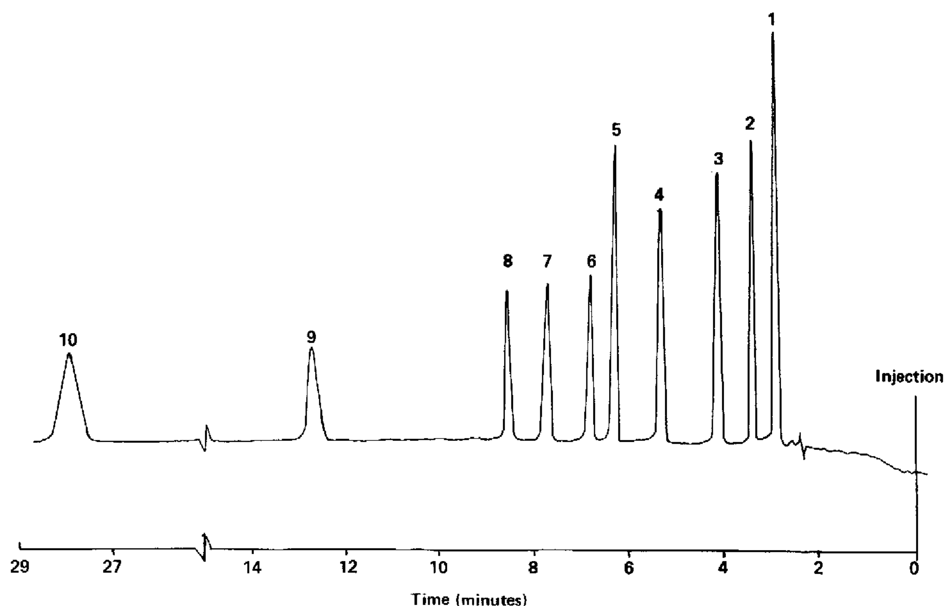

Fig. 2. Reversed-phase HPLC separation of aromatic amines and substituted acetanilides. Conditions as in text. Peaks: 1 = 4-aminophenol; 2 = 4-hydroxyacetanilide; 3 = 3-hydroxyacetanilide; 4 = *p*-anisidine; 5 = aniline; 6 = 4-nitroaniline; 7 = acetanilide; 8 = *p*-phenetidine; 9 = 4-ethoxyacetanilide; 10 = 4-ethylacetanilide.

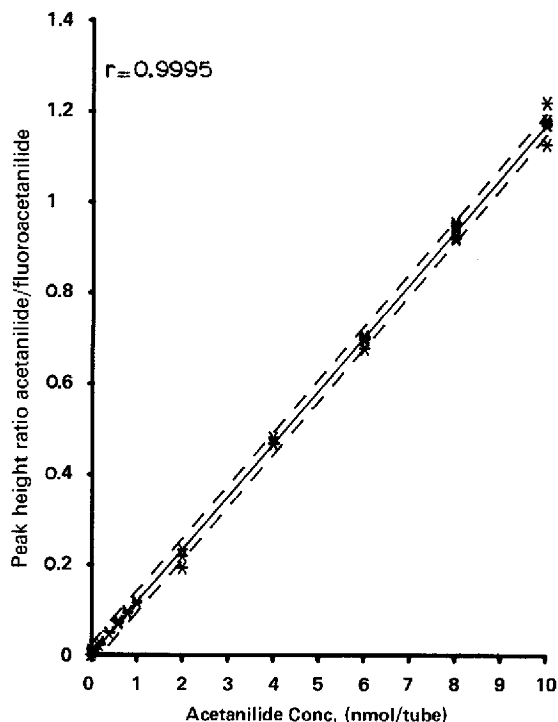

Fig. 3. Calibration graph for the estimation of acetanilide in whole blood using 4-fluoroacetanilide as internal standard. The dashed lines indicate the 95% confidence interval.

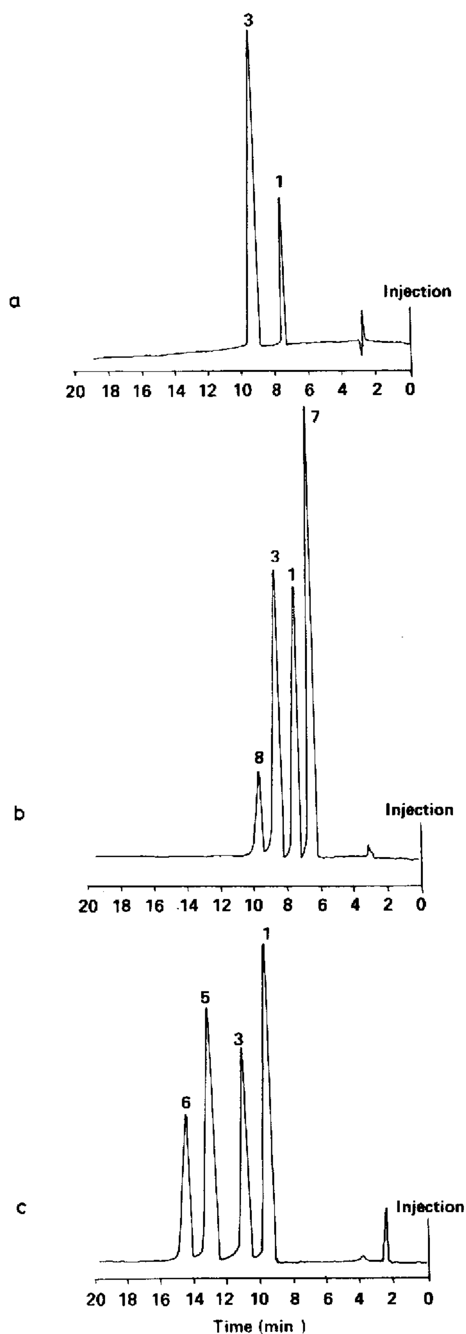

Fig. 4. The separation of some 4-substituted acetanilides on a C_{18} bonded-phase column. Solvent systems: (a) methanol–66.6 m*M* phosphate buffer (pH 7.4) (40:60); (b) ethanol–chloroform–hexane (2.5:20:77.5); (c) tetrahydrofuran–hexane (15:85). The numbers refer to the amides shown in Table I.

TABLE I

SOLVENT SELECTIVITY TOWARDS ACETANILIDE AND ITS SUBSTITUTED DERIVATIVES

Mobile phases: (A) methanol–66.6 m*M* phosphate buffer (pH 7.4) (40:60); (B) ethanol–hexane (5:95); (C) ethanol–chloroform–hexane (3:20:77); (D) tetrahydrofuran–hexane (18:82). HPLC conditions as described in text. The flow-rate for each solvent was 1 ml min^{-1}. N.A. indicates no analysis was performed. The numbers refer to compounds separated in Fig. 4.

Compound	*No.*	*Mobile phase*			
		A	*B*	*C*	*D*
Acetanilide	1	1.0	1.0	1.0	1.0
4-Chloroacetanilide	2	Insoluble	1.0	0.71	0.77
4-Fluoroacetanilide	3	1.41	1.03	1.24	1.11
4-Bromoacetanilide	4	Insoluble	1.03	1.24	1.08
4-Ethoxyacetanilide	5	2.13	1.20	1.0	1.34
4-Methoxyacetanilide	6	0.97	1.40	0.73	1.56
4-Ethylacetanilide	7	4.96	0.88	0.80	0.91
4-Nitroacetanilide	8	N.A.	1.24	1.59	1.35
4-Hydroxyacetanilide	9	0.56	N.A.	4.52	3.47
Acetanilide retention time (min)		7.42	5.76	6.17	6.91

In the studies we have completed so far we have observed a two-fold range in the percentage acetylation of sulphamethazine in eleven subjects at all three concentration levels. The ability of a subject to acetylate sulphamethazine in a reproducible manner has been shown to be constant over a period of ten months, a coefficient of variation (C.V.) of less than 10% being obtained from twenty determinations.

The assay for the acetylation of the aromatic amines has posed some interesting problems. We wish to be able to study the competitive acetylation of amines, such as aniline, ethylaniline, phenetidine and 4-nitroaniline, to determine the effects of structure on the rate and extent of acetylation. To accomplish this, our first thoughts were to use a reversed-phase liquid-chromatographic assay. A separation of several possible substrates and products is shown in Fig. 2. This proved satisfactory for the analysis of acetanilide and phenacetin in our blood samples. The calibration graph for acetanilide, Fig. 3, confirms the linearity of the assay in this range. A typical chromatogram is shown in Fig. 4a.

Since we have not yet devised an efficient extraction procedure for the amine substrates we are only measuring the amides. One problem with the reversed-phase system is that for competitive studies we need to assay acetanilide and 4-ethylacetanilide in the same incubation mixture. Under the chromatographic conditions that allow us to separate acetanilide from the internal standard 4-fluoroacetanilide, the 4-ethylamide derivative is eluted with a long retention time (Fig. 2). We are not in a position to use a gradient separation and have thus studied the selectivity of the bonded-phase column towards a number of 4-substituted acetanilides and compared the reversed-phase separation mode with that in which organic mobile phases are used. The selectivity of the same column towards nine aromatic amides with four different mobile phases is shown in Table I. Fig. 4b and c illustrates how different amides are separated in a relatively short time when only an organic mobile phase is used.

DISCUSSION

Using a modification of the Bratton–Marshall procedure, Mandelbaum-Shavit and Blondheim[7] reported that human blood N-acetyltransferase activity to sulphamethazine was low and undetectable in some cases. Using the HPLC assay we are able to detect activity in all the samples studied at this time. The method allows us to study the acetylation at substrate levels ten times lower than those quoted by the above authors and with much greater specificity. We have shown that plasma alone does not acetylate sulphamethazine or aniline, suggesting the N-acetyltransferase enzymes are in the cellular fraction of the blood. This result has not been reported from human studies, but is in agreement with the data on rabbit blood[8].

The values of V_{max} and K_M quoted are only estimates of these parameters for blood N-acetyltransferase, since the study was not performed at saturating acetyl coenzyme A (AcCoA) concentrations. No AcCoA was added to the incubation mixtures. They will however be a close approximation to the true values, since other studies we have performed with AcCoA suggest that substantial increases in this cofactor are required to influence *in vitro* acetylation. Studies with human blood in vitro show no correlation between a subject's acetylator phenotype and their blood acetylation of either dapsone[9] or *p*-aminobenzoic acid[10]. The low activity of the enzyme and the resulting difficulty in the analysis of its effect on sulphamethazine has until now prevented investigation of blood polymorphism in the acetylation of sulphamethazine. We have shown that acetylation can be monitored *in vitro* with low substrate concentration, and our current work is enabling a comparison of *in vitro* acetylation with acetylator phenotype and a study of acetylation in diabetic subjects.

The production of acetanilide from aniline proceeds at a much faster rate than that for sulphamethazine, and the same effect is obtained with the 4-ethoxy analogue, phenetidine. It is reported that the 4-nitro compound is not acetylated by mammalian liver preparations[11] and thus it is important for us to have data on the incubation of this compound alone and in the presence of those amines which are readily acetylated. The data on the separation of the amides illustrates the flexibility of normal-phase separation. While the reversed-phase system is well suited to the analysis of paracetamol (a broad peak is obtained in the other systems), all our other studies appear to be best served by a separation involving solvent systems C or D (see Table I). We have shown that the amine substrates are eluted before the amides in these systems. While one system is not able to give us complete separation of all the amides we are likely to study, the use of tetrahydrofuran with hexane or the ethanol–chloroform–hexane system appear very promising. An advantage of such systems in our case is that they are most useful for liquid chromatography–mass spectrometry, and in combination with this technique will assist in our study of acetyl group removal and transfer. The separation conditions have not yet been optimised to give the maximum resolution, but this work illustrates how useful this type of separation can be when the matrix that is to be analysed is clearly defined and does not contain highly polar compounds.

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