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THE APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO IN VITRO DRUG METABOLISM STUDIES WITH N-ALKYLARYLAMINES

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SUMMARY

Analytical procedures have been investigated for the separation, detection, identification and quantitation of some metabolites of N-benzyl-4-substituted anilines. Techniques based on gas—liquid chromatography were investigated and found to be unsatisfactory. By the use of reversed-phase high-performance liquid chromatography with gradient and ion-pairing techniques, methods were devised for the simultaneous analyses of a variety of metabolites. The method involves minimum sample work-up (acetonitrile precipitation) and allows easy and prompt analysis in biological media avoiding undue decomposition of unstable metabolites.

INTRODUCTION

In the course of our studies on the in vitro metabolism of nitrogenous xenobiotics, we found N-benzyl-4-substituted anilines are converted to a variety of compounds. Our preliminary experiments with thin-layer chromatographic (TLC) techniques indicated that metabolism involved nitrogen-oxidation, N-dealkylation and ring-hydroxylation, the resulting products having diverse physiochemical characteristics, stability and quantities in which they were produced.

A review of the literature indicated numerous methods were available for the detection and quantitation of in vitro metabolic N-alkylaniline N-dealkylation including colorimetric assays based on the work of Brodie and Axelrod

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[1] and Nash [2], radiometric [3] and gas—liquid chromatographic (GLC) techniques [4]. Similarly, ring-hydroxylated products of N-alkylanilines have been determined by spectrophotometry [5] and more recently by GLC [6—8]. In contrast, studies on N-alkylaniline N-oxidation have suffered from daunting analytical problems due to the inherent instability of these compounds. Previously, non-specific colorimetric analytical procedures, in which the primary N-oxidised product was inferred rather than demonstrated, were used [9], recently a colorimetric assay for primary and secondary arylalkyl hydroxylamino compounds in drug metabolism studies has been described [10]. Because of stability problems, GLC of this type of N-oxidised compounds has proved elusive, but not impossible, since GLC of the trimethylsilyl derivatives of the N-oxidised metabolite of N-methyl-4-aminoazobenzene has been reported [11]. High-performance liquid chromatography (HPLC) has been used in drug metabolism studies since it offers many advantages over more traditional analytical techniques, especially when dealing with particularly labile compounds, recently exemplified with N-hydroxynaphthylamines [12].

Since we wished to study in vitro N-dealkylation, ring-hydroxylation and N-oxidation of N-alkylanilines, we required a method of analysis whereby all these pathways could be followed simultaneously. Results with GLC partly met our criteria, however, analysis of N-oxidation products was unsatisfactory. Therefore, we turned our attention to HPLC and report here our findings for both analytical procedures.

EXPERIMENTAL

Materials

Aniline, 4-chloroaniline, 4-toluidine, N-benzylaniline · HCl, benzaldehyde and heptanesulphonic acid (sodium salt) were purchased from BDH (Poole, U.K.) as the purest grade available. Phenyl-N-(4-substituted)phenylnitrones and N-benzyl-N-(4-substituted)phenylhydroxylamines were synthesised as reported elsewhere [13]. The Schotten-Baumann reaction as described by Vogel [14] was used to prepare the N-benzoyl-4-substituted anilines, all melting points agreed with literature values. N-Benzyl-4-aminophenol was purchased from Eastman (Rochester, NY, U.S.A.). Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). All other solvents used were purchased from BDH and glass distilled prior to use.

Gas—liquid chromatography

A Perkin-Elmer F33 gas chromatograph equipped with a flame ionisation detector and a 0—2.5 mV Hitachi-Perkin-Elmer 159 chart recorder were used. Precoiled 1 m × 6.4 mm O.D. glass columns were packed as follows: Column A, 3% OV-17 on 80—100 mesh AW DMDCS treated Chromosorb G; Column B, 3% OV-1 on 80—100 mesh AW DMDCS treated Chromosorb G.

The columns were conditioned at a temperature 10°C higher than the operating temperature for 48 h. Prior to use, both columns were silanized in situ with 3 × 5 μl of hexamethyldisilazane. Gas pressures were nitrogen 140 kPa, hydrogen 119 kPa and air 175 kPa. Columns were connected to glass-lined inlet ports to minimise the risk of catalytic thermal breakdown. Solutions

of the test compounds were prepared in methanol (1 mg/ml) and 1—2 μl aliquots injected onto the column.

High-performance liquid chromatography

The liquid chromatograph comprised two pumps (Altex Model 110A), coupled to a mixing chamber and interfaced with a microprocessor solvent gradient programmer (Altex Model 420), a syringe loading sample injector valve (Rheodyne 7120) fitted with a 100-μl sample loop. Detection was a variable-wavelength UV spectrophotometer detector (Pye Unicam LC3) and chart recorder (Tekman TE200). The column was a 250 × 5 mm smooth bore seamless annealed 316 stainless-steel tube packed with Spherisorb 5 ODS (HPLC Technology, Wilmslow, U.K.), a microparticulate, 5-μm particle size, reversed-phase material consisting of a C_{18} stationary phase on a silica backbone. A 50 × 5 mm guard column packed with Partisil Co:Pell ODS (Whatman, Maidstone, U.K.), and placed immediately before the analytical column was routinely used. The columns were conditioned by passing appropriate mobile phase through the system for about 2 h prior to use.

Solvents used in the analyses (primarily water and acetonitrile) were filtered through glass fibre grade GF/F paper (Whatman), then degassed under vacuum for 10 min, followed by a 5-min purge with a fine stream of helium obtained using a sintered glass tube. When heptanesulphonic acid in acetic acid was incorporated in the mobile phase, it was added prior to helium purging.

Reference compounds were freshly prepared in methanol (1 mg/ml) and aliquots (10—20 μl) were injected onto the column.

Quantitative analysis of N-benzyl-4-substituted aniline metabolism by HPLC

Authentic samples of reference compounds were prepared in acetonitrile. Aliquots (100 μl) of these were used to spike typical microsomal incubates (3 ml) maintained on ice to give mixtures containing between 5 and 500 nmol of reference compound. The appropriate internal standard (100 nmol in 50 μl acetonitrile) was added and the contents of the flask were quantitatively transferred to a screw-capped tube (10 ml, Sovirel) using acetonitrile (2 ml) (HPLC grade). The tubes were capped, shaken and centrifuged at 10,000 *g* (Sorvall RB2 centrifuge) for 10 min to sediment the protein and particulate matter precipitated by the acetonitrile. The supernatant, an aqueous mixture of acetonitrile (60%), was transferred into fresh tubes and used directly for HPLC analysis. Aliquots (100 μl) were injected onto the column and peak height ratios of the reference compounds to internal standard were plotted against concentration.

RESULTS AND DISCUSSION

Gas—liquid chromatography

The retention times of the compounds are given in Table I. At the oven temperature required to obtain reasonable retention times, aniline and benzaldehyde eluted with the solvent front. Moreover, when aniline and benzaldehyde were injected simultaneously, they produced an additional peak with a retention time of 2.6 min on column A and 1 min on column B. Further

investigation indicated this peak was due to the formation of benzylideneaniline. Of the other compounds examined, N-benzylaniline, N-benzoylaniline and N-benzyl-4-aminophenol chromatographed satisfactorily.

Two peaks were produced when α,N-diphenynitrone was injected onto column A or B, although TLC indicated the reference sample was pure. The peak at retention time 12.6 min on column A and 4.4 min on column B, is probably due to the nitrone, whereas the peak at retention time 2.6 min on column A and 1.0 min on column B, is due to a breakdown product, probably benzylideneaniline. This was confirmed when the chromatographic characteristics of authentic benzylideneaniline were determined. N-Benzyl-N-phenylhydroxylamine was found to yield multiple products on both columns. Of these, the two major peaks had similar retention times to α,N-diphenylnitrone and benzylideneaniline, a third peak corresponded with that obtained for authentic N-benzylaniline.

TABLE I

GAS—LIQUID CHROMATOGRAPHIC SEPARATION OF N-BENZYLANILINE AND SOME POTENTIAL METABOLITES

Compound	Retention time (min)	
	Column A*	Column B**
Aniline	—	—
Benzaldehyde	—	—
N-Benzylaniline	3.0	1.3
Benzanilide	8.4	3.4
N-Benzyl-4-aminophenol	10.2	4.2
α,N-Diphenylnitrone***	12.6, 2.6	4.4, 1.0
N-Benzyl-N-phenylhydroxylamine***	12.6, 2.6, 3.0 + others	4.4, 1.0, 1.3 + others

*Column A: 3% OV-17 on 80—100 mesh AW DMDCS Chromosorb G; 1 m glass; nitrogen, 140 kPa; oven temperature 220°C; injection temperature 250°C.
**Column B: 3% OV-1 on 80—100 mesh AW DMDCS Chromosorb G; 1 m glass; nitrogen, 140 kPa; oven temperature 165°C; injection temperature 200°C.
***Indicates compounds decomposed during chromatography.

Re-examination of α,N-diphenylnitrone chromatographically demonstrated that the products obtained depended upon the amount of nitrone applied. When concentrations of the magnitude expected in metabolic experiments (100 nmol per 100 μl of extracted, concentrated sample) were injected, the peaks at retention time 12.6 min (column A), and 4.4 min (column B) disappeared leaving only the peaks due to benzylideneaniline. This degradation did not appear to be quantitatively reproducible. Therefore, GLC was rejected as a method suitable for the simultaneous determination of the major products of N-benzylaniline metabolism.

High-performance liquid chromatography

By employing mobile phase 1 and the gradient elution programme described in Table II, it was possible to resolve mixtures of N-benzyl-4-substituted

anilines and their potential metabolites. The various compounds eluted discretely, the peak shapes being symmetrical and reproducible, and could be analysed within a reasonable time. The metabolites of N-benzylaniline also chromatographed well under these conditions with the exception of α,N-diphenylnitrone and N-benzyl-4-aminophenol, which tended to co-chromatograph. A complication arose due to the appearance of a minor peak associated with the aminophenol. Aminophenols are easily oxidised in aqueous solution [15], N-benzyl-4-aminophenol is no exception, aqueous solutions of which quickly turn brown on standing, especially in bright light [6]. The oxidised intermediates produced in these reactions are probably of a quinoneimine structure. The minor peak associated with N-benzyl-4-aminophenol could be an ionised species present in equilibrium with the unionised compound, a phenomenon observed with other compounds during HPLC with aqueous mobile phases [16].

TABLE II

HPLC RETENTION TIMES FOR N-BENZYL-4-SUBSTITUTED ANILINES AND THEIR POTENTIAL METABOLITES

Mobile phase 1 (M1): pump A, water (distilled twice); pump B, acetonitrile.
Mobile phase 2 (M2): pump A, 0.005 *M* acetic acid containing 0.005 *M* heptanesulphonic acid (pH of mixture = 3.5 ± 0.1); pump B, acetonitrile containing 0.005 *M* acetic acid + 0.005 *M* heptanesulphonic acid
Programme: solvent gradient, flow-rate 1.5 ml/min; time zero, pump B = 39% of mobile phase, time 8 min, pump B = 39 to 60% of mobile phase in 5 min, time 17 min, pump B = 60 to 39% of mobile phase in 1 min.
Analytical column, 250 × 5 mm packed with Spherisorb 5 ODS; detection, UV 254 nm; temperature, ambient.

Compound	Retention time (min)	
	M1	M2
Aniline	4.5	4.7
Benzaldehyde	6.2	6.4
α,N-Diphenylnitrone	8.4	8.6
N-Benzoylaniline*	10.9	11.1
N-Benzyl-4-aminophenol	8.7	12.7
N-Benzyl-N-phenylhydroxylamine	14.2	14.8
N-Benzylaniline	17.2	18.0
4-Chloroaniline	7.2	ND**
α-Phenyl-N-(4-chloro)phenylnitrone	12.5	ND
N-Benzoyl-4-chloroaniline*	15.2	16.0
N-Benzyl-N-(4-chloro)phenylhydroxylamine	17.0	ND
N-Benzyl-4-chloroaniline	20.0	ND
4-Toluidine	5.7	ND
α-Phenyl-N-(4-tolyl)nitrone	11.2	ND
N-Benzoyl-4-toluidine	13.2	ND
N-Benzyl-N-(4-tolyl)hydroxylamine	14.8	ND
N-Benzyl-4-toluidine	18.8	ND

*Compound used as an internal standard.
**ND = not determined.

Further experiments were performed in which N-benzyl-4-aminophenol was chromatographed in the presence of heptanesulphonic acid (HSA), an ion-pairing agent. The results are presented in Table III. Heptane sulphonic acid did not alter chromatographic properties of α,N-diphenylnitrone or N-benzyl-4-aminophenol and just lowering the pH of the mobile phase with acetic acid appeared to cause breakdown of the aminophenol. However, lowering the pH of the medium with acetic acid enabled an ion-pair to be formed between HSA and N-benzyl-4-aminophenol. The resulting chromatography of the aminophenol is pH-dependent, being optimum at pH 3.5 with negligible change observed for the other potential metabolites (see Fig. 1a and b).

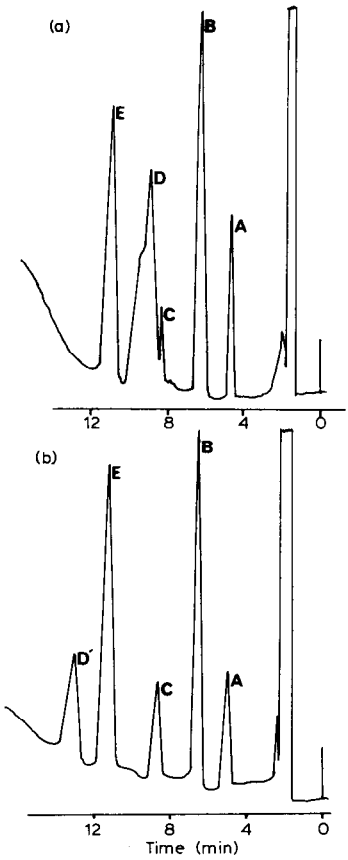

Fig. 1. The influence of heptanesulphonic acid and acetic acid on the HPLC separation of metabolites of N-benzylaniline. (a) Without HSA and acetic acid; mobile phase 1, water—acetonitrile. (b) With HSA and acetic acid; mobile phase 2, water—acetonitrile with 0.005 *M* HSA and 0.005 *M* acetic acid added to each component. Peaks: A = aniline; B = benzaldehyde; C = α,N-diphenylnitrone; D = N-benzyl-4-aminophenyl; D′ = N-benzyl-4-aminophenol/HSA ion pair; E = benzanilide.

Quantitative analysis of metabolites of N-alkyl-4-substituted anilines using HPLC

Once optimum chromatographic conditions were established, calibration graphs were obtained for the potential metabolites with the exception of

TABLE III

EFFECT OF HEPTANE SULPHONIC ACID AND pH ON THE HPLC OF THE METABOLITES OF N-BENZYLANILINE

See Table II for details of programme and mobile phase 1.

Mobile phase	Retention time (min)				
	Aniline	Benz-aldehyde	α,N-Diphenyl-nitrone	N-Benzyl-4-aminophenol	Benz-anilide
Mobile phase 1	4.5	6.2	8.4	8.7	10.9
Mobile phase 1 + 0.005 *M* HSA + 0.001 *M* acetic acid (pump A, pH = 3.8)	4.6	6.3	8.6	10.2	11.1
Mobile phase 1 + 0.005 *M* HSA + 0.0025 *M* acetic acid (pump A, pH = 3.65)	4.6	6.3	8.6	12.2	11.1
Mobile phase 1 + 0.005 *M* HSA + 0.005 *M* acetic acid (pump A, pH = 3.5)*	4.7	6.4	8.6	12.7	11.1
Mobile phase 1 + 0.005 *M* HSA	4.5	6.2	8.4	8.7	10.9

*Equivalent to mobile phase 2 in Table II.

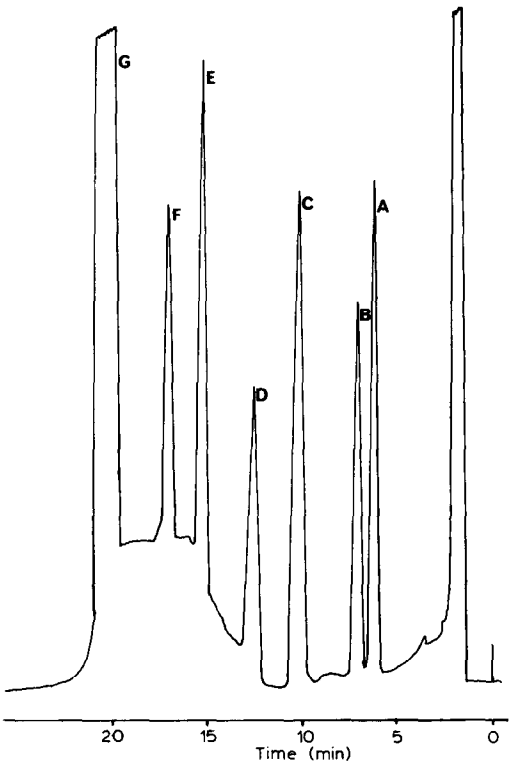

Fig. 2. HPLC separation of N-benzyl-4-chloroaniline and its metabolites. Peaks: A = benzaldehyde; B = 4-chloroaniline; C = benzanilide (internal standard); D = α-phenyl-N-(4-chloro)phenylnitrone; E = N-benzoyl-4-chloroaniline; F = N-benzyl-N-(4-chloro)phenylhydroxylamine; G = N-benzyl-4-chloroaniline.

N-benzyl-N-(4-tolyl)hydroxylamine. This compound, which was the least stable disubstituted hydroxylamine synthesised, appeared to undergo almost total degradation in aqueous media at pH 7.4 to yield numerous products, predominantly the nitrone, together with benzaldehyde and the parent amine. Consequently, should N-benzyl-N-(4-tolyl)hydroxylamine be metabolically formed, it would almost certainly undergo spontaneous decomposition whilst in its incubation media. Therefore, it was not included in the spiked solutions.

Some of the compounds tested have low aqueous solubility and problems of co-precipitation (with protein) were envisaged during sample work-up. However, careful handling, including keeping samples cool, but avoiding temperatures of less than 10° C, ensuring minimum analytical delay and the storage of samples in the dark prior to analysis, avoided problems. Plots of amounts of compound present in the mixture against peak heights recorded after chromatography demonstrated linear correlations with coefficients greater than 0.997. A chromatogram showing the separation of N-benzyl-4-chloroaniline and its metabolites in a spiked microsomal incubate is given as an example in Fig. 2.

Complete separation of N-benzyl-4-substituted anilines and their potential metabolites in biological media has been achieved by reversed-phase gradient elution HPLC. In one instance, separation was facilitated by the use of an ion-pairing technique. Our procedures are relatively straightforward and allow diect analysis of products of in vitro metabolism. The methods described avoid the ambiguity associated with former analytical methods and have been used to investigate the enzymology involved in the in vitro metabolism of N-benzyl-4-substituted anilines [16].

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