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Note

Analysis of amine metabolites by high-performance liquid chromatography on silica gel with a non-aqueous ionic eluent

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Analyses of tertiary amines and their oxidative metabolites from *in vitro* incubation mixtures constitute an important part of human drug metabolism studies because a large number of drugs are tertiary amines. Quantification of amines has relied on gas chromatography and reversed-phase high-performance liquid chromatography (HPLC) but neither method is useful when highly unstable or non-polar metabolites are present. For example, a tertiary amine such as zimeldine (I, Fig. 1) is extensively metabolized in humans and animals [1–3]. It is extremely difficult to quantify highly polar metabolites such as nitrone (IV) as well as non-polar aldehyde (V) in a single chromatographic procedure. Dimethylaniline, chlorpheniramine and brompheniramine are additional examples of ma-

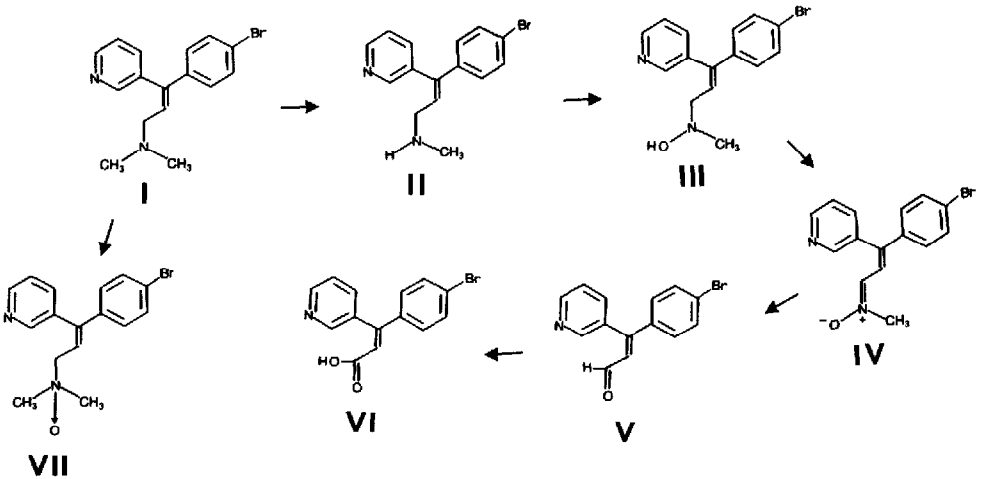

Fig. 1 Overall biotransformation of zimeldine (I) to norzimeldine (II), norzimeldine hydroxylamine (III), norzimeldine nitrone (IV), aldehyde (V), acrylic acid (VI) and zimeldine N-oxide (VII)

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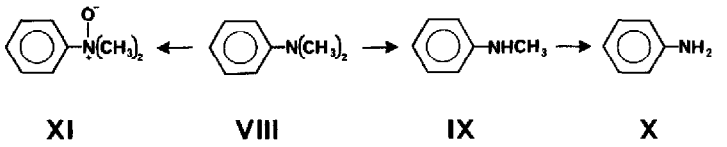
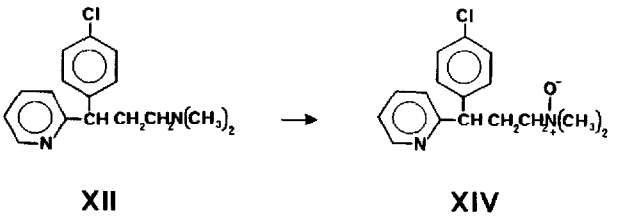


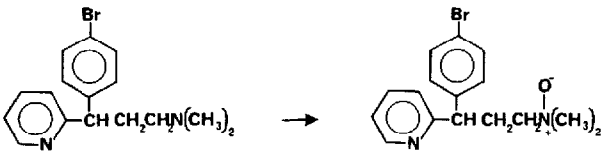
XI VIII IX X

Fig 2 Overall biotransformation of N,N-dimethylaniline (VIII) to N-methylaniline (IX), aniline (X) and N,N-dimethylaniline N-oxide (XI)

terials biotransformed to highly polar tertiary amine N-oxide metabolites (Figs. 2 and 3) [4]. We have developed an ion-pair HPLC method for the analysis of tertiary amines and their oxidative metabolites. This method allows for direct analysis of dichloromethane extracts without the need for derivatization. The procedure has several advantages over current assays of tertiary amines and their metabolites including speed of assay and ability to identify metabolites of widely varying structure from *in vitro* incubation mixtures.



XII XIV



XIII XV

Fig 3. N-Oxygenation of chlorpheniramine (XII) and brompheniramine (XIII) to chlorpheniramine N-oxide (XIV) and brompheniramine N-oxide (XV), respectively.

EXPERIMENTAL

Apparatus

Chromatography was performed on a Beckman (San Jose, CA, U.S.A.) Model 110A and 110B solvent delivery system, Model 421 controller, a Kratos Model 757 Spectroflow UV detector (Ramsey, NJ, U.S.A.), and a Hewlett Packard Model 13390A integrator (Santa Clara, CA, U.S.A.). A 5-μm, 25 cm × 4.5 mm AXXIOM silica column (Richard Scientific, Novato, CA, U.S.A.) was used for all separations.

Materials

Zimeldine and its metabolites were synthesized as previously described [1,5]. Dimethylaniline, N-methylaniline and aniline were obtained from Aldrich (Milwaukee, WI, U.S.A.). Dimethylaniline N-oxide was synthesized as previously described [4]. Chlorpheniramine and brompheniramine were gifts from Schering (Bloomfield, NJ, U.S A.) and their N-oxides were synthesized by a method previously described [4].

Extraction

The extraction of authentic metabolites from aqueous solution or metabolic mixtures (to be reported in subsequent publications) was accomplished by the addition of 4 volumes of cold dichloromethane and extracted for 3 min, and the mixture was centrifuged at 1000 *g* for 5 min. The dichloromethane layer was removed and passed through a nylon filter, evaporated to dryness and the residue was dissolved in 0.5 ml of methanol.

Chromatography

In all runs, 15 μl of the methanolic solution containing the metabolites were injected. The HPLC mobile phase, a mixture of, on the one hand, 60% aqueous perchloric acid and, on the other hand, methanol (or a methanol–acetonitrile mixture) in the proportions 0.2–0.8:1000 (v/v) was pumped through the column at a flow-rate of 1.0 ml/min.

RESULTS

Ion-pair desorption chromatography

Tertiary amines and tertiary amine metabolites often fail to give highly reproducible chromatographic separations. For example, tertiary amine N-oxides or secondary hydroxylamines may completely change their order of elution with respect to the parent tertiary amine as analyzed by reversed-phase HPLC. It is often times unpredictable what structural or chemical feature determines the reversed-phase chromatographic properties and retention volumes of tertiary amine metabolites. Therefore, we developed a sensitive HPLC method for this general class of metabolites based on non-aqueous ion-pair chromatography on silica.

The solutes of interest possess ionizable amine groups suggesting that their retention may be selectively enhanced by the addition of an oppositely charged, anionic pairing ion to the mobile phase. The retention of all amine solutes was found to increase with increasing concentration of 60% perchloric acid (0.001–0.0001 *M*) in a mobile phase consisting of methanol–perchloric acid. No changes in elution order were observed with changes in the perchloric acid concentrations. The optimum conditions for the separation and quantification of some representative tertiary amine metabolites in metabolic extracts are summarized in Ta-

TABLE I

CHROMATOGRAPHIC CONDITIONS

Chromatography was performed with a silica column, UV detection at 260 nm, flow-rate of 1 0 ml/min, 25°C and sample volume of 15 μl.

Compound	Mobile phase	Retention time (min)
E-I, III, IV	CH_3CN–CH_3OH–$HClO_4$ (57.4·42.5:0.05)	13 9, 6 4, 8 3
VIII, IX, XI	CH_3OH–$HClO_4$ (99.98.0 02)	13 6, 8 5, 12.8
XII, XIV	CH_3CN–CH_3OH–$HClO_4$ (60 40 0 08)	6.5, 5.3
XIII, XV	CH_3CN–CH_3OH–$HClO_4$ (60·40·0 08)	6 4, 5.4

ble I. The optimization of the chromatographic conditions was performed with perchloric acid–methanol in the mobile phase. It was found that addition of acetonitrile could sometimes improve separation and peak shapes. The studies described herein were performed at ambient temperature (25°C) with a flow-rate of 1.0 ml/min.

Linearity

For the amines examined (I–IV, VII–XV), there was a linear relationship between peak area and concentration of the amines in aqueous solution over the following range: 50–500 ng/ml. The limits of detection, taken to be the peak height corresponding to twice the baseline noise, was found to be 5–20 ng/ml for the tertiary amines or their metabolites.

Recovery and reproducibility

Generally, amounts (1.5 μg) of each compound added to buffer or buffer containing inactive protein were determined to be recovered in 90–100%. We assessed the long-term reproducibility of analysis of tertiary amines and their metabolites by comparing results obtained with a new silica column (described above) with an old silica column (*i.e.* one that had been in use two to three years). In general, the old silica gel column required higher concentrations of perchloric acid in order to effect the same chromatographic separation. However, even silica gel columns that had been used extensively in the past provided excellent chromatograms with sharp peaks, identical elution order and similar solute peak separation and resolution.

Amine examples

The HPLC method was applied to the separation of a variety of tertiary amines and their oxidative metabolites. The metabolism of hydroxylamine (III) was investigated and the tertiary amine *E*-zimeldine was added as an internal

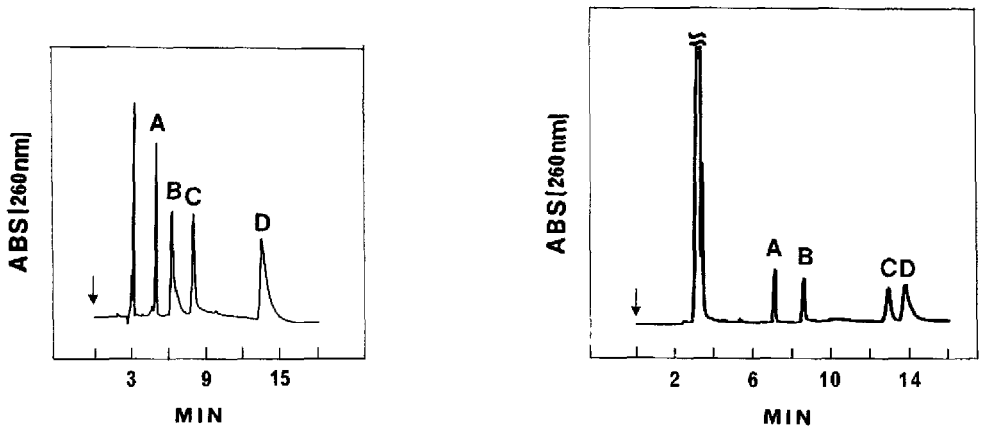


Fig. 4 Liquid chromatogram of a mixture of possible metabolites of hydroxylamine (III) after addition of internal standard Peaks: A = aldehyde (V; 9 ng), B = nitrone (IV, 46 ng); C = hydroxylamine (III; 35 ng), D = *E*-zimeldine (I, 137 ng) The arrow indicates the time of injection

Fig 5 Liquid chromatogram of a mixture of possible metabolites of N,N-dimethylaniline. Peaks. D = N,N-dimethylaniline (VIII; 200 ng), B = N-methylaniline (IX, 171 ng), A = aniline (X; 171 ng), C = N,N-dimethylaniline N-oxide (XI; 304 ng).

standard (Fig. 1). As shown in Fig. 4, the hydroxylamine (III) is readily separated from the nitrone (IV) and aldehyde (V) metabolites. Another example of HPLC separation of a tertiary amine and its metabolites is of the aromatic amine dimethylaniline and is shown in Fig. 5. Dimethylaniline (VIII) is readily separated from its potential metabolites: tertiary amine N-oxide (XI), secondary amine (IX) and primary amine (X). The aliphatic tertiary amines chlorpheniramine (XII) and brompheniramine (XIII) are readily separated from their aliphatic tertiary amine N-oxide metabolites XIV and XV, respectively, as shown in Fig. 6a and b, respectively.

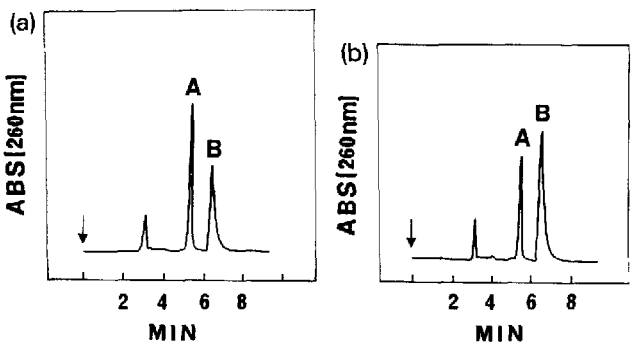


Fig. 6. (a) Liquid chromatogram of (A) chlorpheniramine N-oxide (XIV, 52 ng) and (B) chlorpheniramine (XII, 67 ng). (b) Liquid chromatogram of (A) brompheniramine N-oxide (XV, 36 ng) and (B) brompheniramine (XIII, 100 ng).

DISCUSSION

Because of the large number of amine-containing drugs used in medicine and because of the large number of potential oxidized metabolites, an accurate and reproducible method is required for the identification of tertiary amines and their metabolites in dichloromethane extracts of metabolic reactions. We have developed a method for amine assay based on non-aqueous ion-pair eluent silica gel HPLC analysis [6]. This method allows for direct analysis of aqueous samples or incubation mixtures without the need for derivatization. The analysis has the further advantage of using a simple HPLC eluent which stabilizes the solutes under conditions which affords ready separation. The HPLC procedure described here is sensitive, rapid and simple. The assay requires no derivatizations of products and avoids the use of radioactive substrates. The general applicability of this method for clinical analysis of amine-containing samples is under investigation.

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