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RESOLUTION OF ENANTIOMERIC MIXTURES OF PRIMARY ALIPHATIC HYDROXYLAMINES AND AMINES BY GAS CHROMATOGRAPHY

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SUMMARY

A method is described for the analysis of enantiomeric mixtures of primary aliphatic amines and the corresponding N-hydroxylamine metabolites. Optically active substrates are converted to diastereomeric derivatives by reaction with a chiral acylating agent and the products are separated by gas–liquid chromatography, yielding four peaks corresponding to the resolved amines and hydroxylamines. Optical composition can be calculated and concentrations of isomers determined at levels approaching 1 μg/ml. The absolute configuration of the resolved hydroxylamines has been determined.

INTRODUCTION

Acetophenone oxime is reductively metabolized by mammalian-liver microsomal enzymes to the corresponding hydroxylamine (N-hydroxy-α-methylbenzylamine) and amine (α-methylbenzylamine)[1,2]. Reduction introduces an asymmetric carbon atom into the molecule, so that the products are enantiomeric mixtures of unknown optical composition. Recently, we described a gas chromatographic (GC) method for monitoring acetophenone oxime and its reductive metabolites in liver homogenates[3]. Resolution of optical mixtures of α-methylbenzylamine was achieved by initial separation of amine from hydroxylamine by extraction, conversion of the enantiomeric amines into diasteromeric amides by reaction with N-trifluoroacetyl-(*S*)-prolyl chloride and subsequent gas–liquid chromatography (GLC) of the amides. Attempts to resolve enantiomeric N-hydroxy-α-methylbenzylamines by a similar reaction involving formation of diastereomeric hydroxamic acids were unsuccessful. GC analysis of the "derivatized" mixture indicated that the hydroxylamine was converted into the same products as were formed on reaction with α-methylbenzylamine. For this reason, quantitative separation (extraction) of amine and hydroxylamine was required before derivatization of the amine.

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Here, we describe a modification of the reported procedure which permits total specific analysis of optical mixtures of amines and hydroxylamines without prior separation from each other.

EXPERIMENTAL

Apparatus

The GLC was carried out on a Beckman GC-45 chromatograph equipped with a flame-ionization detector; mass spectrometry was performed using a Varian CH-5 instrument, with the ionizing potential maintained at 70 eV.

Reagents

N-Trifluoroacetyl-(*S*)-prolyl chloride (TPC) (0.1 *M* solution in chloroform) was purchased from the Regis Chemical Co. (Chicago, Ill., U.S.A.); *R*(+), *S*(−) and *RS*(±)-forms of α-methylbenzylamine were obtained from the Eastman Chemical Co. (Rochester, N.Y., U.S.A.); and N-hydroxy-α-methylbenzylamine was synthesized by reduction of acetophenone oxime with sodium cyanoborohydride[4].

Derivatization

Microsomal suspensions (to which 0.001% of EDTA had been added) containing amine and hydroxylamine (total 1.25 μmoles) were extracted with an equal volume of chloroform. The chloroform solution was evaporated to dryness, and the residue was dissolved in 250 μl of chloroform. Then 25 μl (2.5 μmoles) of TPC solution were added, followed, after 1 min, by 2.5 μmoles of triethylamine. One minute later, 250 μl of sodium chloride-saturated 1 *M* hydrochloric acid were added, and the mixture was shaken vigorously for 1 min; the phases were separated, and 2 μl of the chloroform solution were subjected to GLC.

Chromatography

The resulting mixture of diastereomeric amides and hydroxamic acids was chromatographed isothermally at 200° on a 5.5-ft. × 1/8-in. glass column packed with 3% of OV-17 coated on Gas-Chrom Q (100–120 mesh). The injector temperature was 250°, the detector was at 250°, and the nitrogen carrier gas flow-rate was 30 ml/min. For collection of effluent, a stream splitter that gave a 6:1 split in favor of the collector was used.

RESULTS AND DISCUSSION

Resolution of enantiomeric mixtures of α-methylbenzylamine and N-hydroxy-α-methylbenzylamine was achieved by their conversion into diastereomeric amides and hydroxamic acids, respectively, by reaction with TPC[3,5,6]. The diastereomers were then separated by GLC on an OV-17 column. Chromatographic analysis yielded four peaks (see Fig. 1); those with retention times of 2.2 and 2.6 min corresponded to the resolved amides, and the resolved hydroxamic acids were eluted at 3.0 and 3.9 min (see Table I). Conversion of both amines and hydroxylamines into acylated derivatives at room temperature was quantitative, and the reaction was complete in 1 min. Maximum yields were obtained when the ratio of acid chloride to acylable

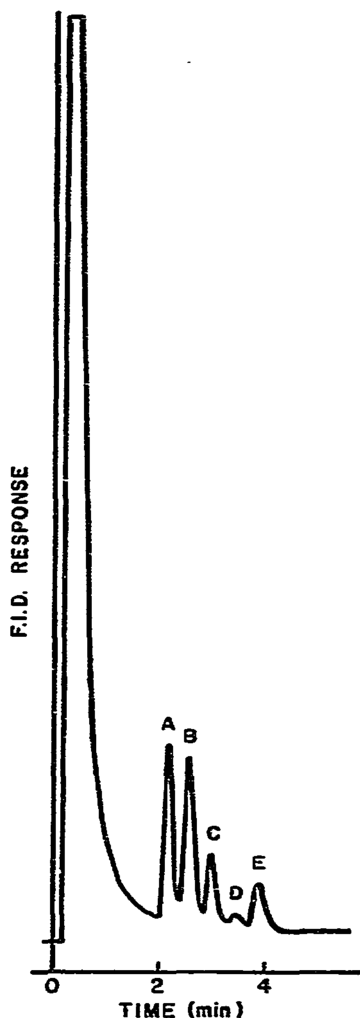

Fig. 1. Chromatogram of the TPC-amides of the (*R*)- and (*S*)-forms of α-methylbenzylamine (A and B, respectively) and the TPC-hydroxamic acids of the (*R*)- and (*S*)-N-hydroxy-α-methylbenzylamine (C and E, respectively); D represents an impurity. Each compound (10 ng) was separated by using the procedure described in the text.

material was 2:1. The resulting amides and hydroxamates were stable in chloroform solution and thermally stable under the conditions used for GLC. Chromatography of the acylated hydroxylamine showed no breakdown (*i.e.*, extraneous peaks) to the corresponding amide, amine or other product. The relationship between peak area and resolved amine or hydroxylamine concentration were linearly related over a wide concentration range (see Table I). The goodness of fit of the calibration graphs obtained over this concentration range was determined by least-squares analysis of data points. The sensitivity, of the detector to the amides was five times better than that for the corresponding hydroxamic acids. Further, the response factors for the diastereomers themselves differed by as much as 30%.

TABLE I

STANDARD GRAPHS FOR GLC OF ENANTIOMER MIXTURES OF ACETOPHENONE OXIME METABOLITES

Compound	*Retention time* (min)*	*Sensitivity limit** (μg/ml)*	*Linear slope*	*Regression parameters****	
				Intercept	*Correlation coeff.*
(*R*)-α-Methylbenzylamine	2.2	1.0	0.086	−0.009	0.999
(*S*)-α-Methylbenzylamine	2.6	1.0	0.118	−0.015	0.999
(*R*)-N-Hydroxy-α-methylbenzylamine	3.0	5.0	0.018	−0.013	0.999
(*S*)-N-Hydroxy-α-methylbenzylamine	3.9	5.0	0.014	−0.003	0.999

* Analysis performed as described in Experimental.

** Based on 1-μl injections.

*** Each curve represents triplicate analyses at six concentrations, ranging from 1 to 60 μg/ml for the amine and from 5 to 180 μg/ml for the hydroxylamine. Graphs were prepared by plotting peak area *vs.* concentration of analyte.

The absolute configurations of the amines were determined by comparison of GLC characteristics for the resolved compounds with those for authentic samples of appropriately derivatized optically pure amines[3]. These peaks were, however, contaminated with small amounts of the amide formed by reaction with the (*R*)-form of TPC present as an impurity (6.75%) in the resolving agent. Optical compositions could still be determined, however, after correction for this amount[3,7].

The absolute configuration of the resolved hydroxylamines was determined from samples (*ca.* 10 μg) of the derivatized hydroxylamines, which were separated by GLC and isolated from the column effluent (corresponding to peaks with retention times of 3.0 and 3.9 min). Each compound was dissolved in 95% ethanol and reduced for 12 h in a hydrogen atmosphere (1 atm.) in the presence of a catalyst (5% platinum oxide on charcoal). Authentic samples of optically pure amine and corresponding TPC-amide did not racemize when subjected to identical reaction conditions. The reduced products were then again derivatized with TPC and re-chromatographed. The hydroxamic acid with a retention time of 3.9 min now gave a peak at 2.6 min, corresponding to the amide derived from (*S*)-α-methylbenzylamine. The hydroxamate with a retention time of 3.0 min yielded a reaction product that was eluted in 2.0 min and was identified as the TPC-amide of (*R*)-α-methylbenzylamine by comparison with an authentic sample. Thus, the peak emerging at 3.0 min corresponds to the hydroxamic acid formed from (*R*)-N-hydroxy-α-methylbenzylamine and the peak at 3.9 min is derived from the (*S*)-enantiomer.

The structures of the derivatives were determined from the mass spectra of the GLC effluent corresponding to the four peaks of interest. The peaks due to the two derivatized amines yielded identical mass spectra; the resolved hydroxylamine bands also generated spectra indistinguishable from one another. The electron-impact mass spectrum of the derivatized amines showed a molecular ion (*m/e* 314; $M^+ = C_{15}H_{17}N_2O_2F_3$) corresponding to the TPC-amide and a base peak at *m/e* 120 ($M^+ - C_7H_7NO_2F_3$) attributable to loss of the trifluoroacetylprolyl residue, indicating that the derivative was the TPC-amide of α-methylbenzylamine. The spectrum of the derivatized hydroxylamines showed a molecular ion (*m/e* 330; $M^+ = C_{15}H_{17}N_2O_3F_3$)

corresponding to monoacylation of the hydroxylamine by TPC. A prominent fragment ion at *m/e* 313 (M^+ — OH) resulting from loss of a hydroxyl-group was present, suggesting that acylation occurred on the nitrogen atom. No peak was observed at *m/e* 120 corresponding to the loss of trifluoroacetylproline (M^+ — $C_7H_7NO_3F_3$) from the parent, which would be expected if acylation had occurred on the oxygen atom. The derivative thus appeared to be a single product, N-trifluoroacetylprolyl-N-hydroxy-α-methylbenzylamine.

CONCLUSIONS

A method is described for the analysis, resolution and quantitation of enantiomeric mixtures of aliphatic primary amines and the corresponding hydroxylamines, based on their acylation with a chiral reagent and subsequent GLC.

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REFERENCES

1 J. Hes and L. A. Sternson, *Drug Metab. Disp.*, 2 (1974) 345.
2 L. A. Sternson and J. Hes, *Pharmacology*, 13 (1975) 234.
3 L. A. Sternson, F. Hincal and S. J. Bannister, *J. Chromatogr.*, 144 (1977) 191.
4 R. F. Borch, M. D. Bernstein and H. D. Durst, *J. Amer. Chem. Soc.*, 93 (1971) 2897.
5 B. Halpern and Y. W. Westley, *Chem. Commun.*, (1966) 34.
6 E. Gordis, *Biochem. Pharmacol.*, 15 (1966) 2124.
7 TPC Product Literature, Regis Chemical Co., Chicago, Ill., U.S.A.