Journal of Chromatography, 144 (1977) 191–200

CHROM. 10,202

GAS CHROMATOGRAPHIC ANALYSIS OF ACETOPHENONE OXIME AND ITS METABOLITES

LARRY A. STERNSON*, FILIZ HINCAL** and STEVE J. BANNISTER
Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kan. 66044 (U.S.A.)
(Received April 27th, 1977)

SUMMARY

A gas–liquid chromatographic (GLC) method has been developed for monitoring the metabolic reduction of acetophenone oxime or oxidative metabolism of the corresponding amine, α-methylbenzylamine in liver homogenates. The oxime, amine, N-hydroxy-α-methylbenzylamine and acetophenone are quantitatively determined after GLC separation of components with temperature programming on an SP-2401-DB-coated column. The first three compounds were silylated with N,O-bis(trimethylsilyl)-acetamide prior to chromatographic analysis to enhance the stability and improve the chromatographic properties of these components. The effluent gas was monitored with flame ionization detection, and permitted quantitation of components at sub-μg/ml levels with reproducibility between injections of ±2%. The optimal composition of enantiomeric mixtures of (*R*,*S*)-α-methylbenzylamines formed during metabolic reduction of acetophenone oximes were determined by conversion to diastereomeric amides and subsequent GLC analysis.

INTRODUCTION

Interest in the metabolism of oximes was generated by the discovery that primary aliphatic amines (*e.g.* amphetamine) are oxidatively metabolized to oximes which can then be converted to the corresponding ketone by enzymatic or chemical hydrolysis. The oxidation proceeds by initial N-oxidation to yield hydroxylamines[1–3] which are dehydrogenated to yield the oxime, or alternatively via C-oxidation to generate a transient carbinolamine[4–6] which eliminate water to form an imine which is N-hydroxylated to provide the oxime. The metabolism of the resultant oximes has been similarly studied[7,8]. The oxime is reduced by hepatic enzymes to produce the corresponding hydroxylamine and amine.

Several gas and thin-layer chromatographic methods have been reported for monitoring these reactions and have been reviewed by Beckett and Haya[9]. The hydroxylamine has been shown to be quite unstable both in solution and when subject-

* To whom correspondence should be addressed.
** On leave from the University of Hacettepe, Ankara, Turkey.

ed to the thermal stresses of gas–liquid chromatographic (GLC) analysis. The various factors for handling these compounds in solution have been described recently by Beckett *et al.*[10]. Breakdown of the hydroxylamine during GLC analysis has required prior derivatization of the hydroxylamine. Similarly, the amine and oxime have proven to require derivatization to enhance their stability for GLC analysis as well as to reduce the severe tailing observed during chromatography of these compounds. None of these methods is capable of simultaneously analyzing for the ketone, oxime, hydroxylamine and amine. Furthermore, reduction of the oxime generates an asymmetric carbon atom; therefore, a method capable of determining the optical purity of the metabolically derived hydroxylamine and amine was desired.

A GLC method is described for the simultaneous analysis of acetophenone oxime and its metabolites N-hydroxy-α-methylbenzylamine, α-methylbenzylamine and acetophenone from biological fluids. A means for resolving the enantiomeric amine metabilotes is presented using a procedure similar to that previously described by Martin *et al.*[11].

EXPERIMENTAL

Apparatus

Gas chromatography was carried out on a Varian 2100 chromatograph equipped with flame ionization detector (FID). High-performance liquid chromatography (HPLC) was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000-A solvent delivery system, Model U-6K septumless injector and Model 440 dual-channel absorbance detector operated at 254 nm.

Reagents

Acetonitrile and methanol were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) N,O-bis(trimethylsilyl)acetamide (BSA) was obtained from Pierce (Rockford, Ill., U.S.A.). N-trifluoroacetyl-(*S*)-prolyl chloride (TPC), 0.1 *M* solution in chloroform containing 6.95% (*R*)-isomer, was purchased from Regis (Chicago, Ill., U.S.A.). (*R*)-(+)-, (*S*)-(−)- and (*R,S*)-(±)-α-methylbenzylamine were obtained from Aldrich (Milwaukee, Wis., U.S.A.) and acetophenone and acetophenone oxime were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). N-Hydroxy-α-methylbenzylamine was synthesized by reduction of acetophenone oxime with diborane[12].

Extraction

Microsomal suspensions (to which 0.001% EDTA had been added) containing oxime, hydroxylamine, amine and ketone were adjusted to pH 4, deaerated with nitrogen, and extracted with an equal volume of chloroform. The organic phase was retained for further analysis. The aqueous layer was adjusted to pH 11.5, and extracted with an equal volume of chloroform.The organic phase was removed and either combined with the chloroform solution obtained from the pH 4 extraction or processed separately.

To determine the extraction efficiency for partitioning of amine and hydroxylamine into chloroform, both compounds were dissolved in acetate buffer (0.2 *M*; pH 4, containing 0.001% EDTA) to the extent of 0.75 mg/ml. The solutions were then

carried through the 2-step extraction just described. 4-Fluorophenol (0.5 mg/ml) was added to each separated phase as internal standard and the mixtures analyzed by HPLC, Separation of α-methylbenzylamine, acetophenone oxime and N-hydroxy-α-methylbenzylamine utilized a μBondapak C_{18} column (4 mm O.D. × 30 cm) (Waters Assoc.) operating at 2.0 ml/min with methanol–water containing potassium hydrogen phosphate (7.1 g/l) adjusted to pH 4.2 (30:70) as mobile phase. With these conditions the retention volumes of amine, hydroxylamine, and oxime were 5.6, 8.4 and 30.8 ml, respectively. Calculation of the amount of amine or hydroxylamine in either aqueous or organic phase was made by drawing an aliquot from either extraction phase, injecting it into the chromatograph and comparing the peak height (relative to internal standard) for the component of interest with standard curves constructed for the amine and hydroxylamine. These standard curves were generated from HPLC analysis of solutions of known concentration of amine and hydroxylamine (in the concentration range 0.25–1.0 mg/ml) prepared both in chloroform and 0.2 *M* acetate (pH 4) buffer. All measurements were made relative to a constant amount of 4-fluorophenol (internal standard) and made in duplicate.

Silylation procedure

The chloroform layers obtained after extraction of amine, hydroxylamine, oxime and ketone were evaporated to dryness. An approximate 60-fold molar excess of BSA (20-fold excess for each derivatizable compound) was added to the mixture, followed by 3 parts of dry acetonitrile (for each part of BSA). The mixture was shaken vigorously for 1 min, and then heated at 60–70° for 30 min. N,N-Dimethylaniline was added as internal standard and the mixture diluted to volume with dry acetonitrile.

Gas chromatographic analysis

Mixtures containing ketone and silylated amine, hydroxylamine and oxime were separated on a 6 ft. × $^1/_4$ in. glass column packed with GP 5% SP-2401-DB coated on Supelcoport (100–120 mesh). Nitrogen, used as carrier gas, flowed at a rate of 30 ml/min. Mixtures were separated with the aid of a temperature program: 75° for 2 min, then heated at 10°/min to 125° where the temperature was maintained for 1 min. The injector and detector temperatures were 140° and 160°, respectively. The amount of each component in a mixture was determined as the ratio of its peak height relative to a known amount of N,N-dimethylaniline (internal standard). Concentrations were determined from calibration curves constructed for each compound, prepared by GLC analysis of standard solutions of silylated amine, hydroxylamine, and oxime and underivatized ketone, each at 13 different concentrations in the range of 0.3–500 μg/ml. Curves were prepared for each compound by plotting concentration *vs.* peak height ratio of material. All measurements were made in triplicate and subjected to linear regression analysis (Table I).

Determination of the optical composition of (R,S)-α-methylbenzylamine mixtures

The chloroform layer obtained from the pH 11.5 extraction was evaporated to dryness and the residue dissolved in 250 μl chloroform. Twenty-five microliters (2.5 μmoles) of TPC solution were added to the solution (containing *ca.* 1.25 μmole of acylable compound), followed in 1 min by the addition of 2.5 μmoles of triethylamine.

TABLE I

STANDARD CURVES FOR GLC ANALYSIS OF ACETOPHENONE OXIME METABOLITES

Each curve represents triplicate analysis of each compound at 10 or 13 concentrations in the range of 0.3–500 μg/ml. Analysis performed as described in Experimental. Sensitivity is based on 1-μl injections.

Compound	*Retention Time*	*Sensitivity limit (μg/μl)*	*Linear regression parameters**		
			Slope	*Intercept*	*Correlation coefficient*
α-Methylbenzylamine	4.9	8.0	2.030	−0.001	0.999
Acetophenone	5.5	0.3	1.650	−0.002	0.998
N-Hydroxy-α-methylbenzylamine	6.3	0.3	2.259	0.002	0.999
Acetophenone oxime	6.7	0.3	2.018	0.004	0.999

* Amount of each compound determined as ratio of peak height of compound relative to internal standard N,N-dimethylaniline.

One minute later, 250 μl of sodium chloride-saturated 1 *N* HCl solution were added and the mixture was shaken vigorously for 1 min. After phase sepatation, 2.5 μl of the chloroform solution were subjected to GLC analysis. The resulting diastereomeric amides were chromatographed on a 4 ft. × $^1/_8$ in. glass column packed with 3% OV-17 coated on Gas-Chrom Q (100–120 mesh). Components were separated (with nitrogen carrier gas flowing at 30 ml/min) isothermally (temperatures: injector, 250°; column, 200°; FID, 260°).

RESULTS

The analysis of acetophenone oxime, its metabolites, N-hydroxy-α-methylbenzylamine and α-methylbenzylamine, and degradation product, acetophenone, was accomplished in three stages: (1) extraction from biological fluid (in the present study rat liver homogenate), (2) chemical derivatization of the oxime, amine and hydroxylamine, and (3) GLC analysis of the mixture with flame ionization detection of the effluent gas.

Extraction

Extraction from biological fluid was carried out in two steps. The ketone, oxime and hydroxylamine were extracted into chloroform after adjustment of the pH to 4. At this pH the amine ($pK_a \approx 9.4$) exists completely in the protonated form and remains in the aqueous phase. No amine was detected in the organic phase after extraction; while at this pH the hydroxylamine ($pK_a \approx 4.8$) was quantitatively (100%) extracted into chloroform. After readjustment of the pH to 11.5, the amine was quantitatively extracted into chloroform. The chloroform extracts were then either combined or analyzed separately. Solvent was removed prior to the next stage in the analysis sequence. At pH 4, only 0.8% of the hydroxylamine was converted to oxime in 4 h. At pH 11.5, however, the hydroxylamine proved unstable, being converted to oxime. Similarly, the presence of EDTA (0.001%) was required to maximize the stability of the hydroxylamine[10]. Extraction efficiency was determined by analyzing the organic phase by a HPLC method capable of discriminating among amine, hydro-

xylamine and oxime, a requirement not met by simple spectrophotometric monitoring of reaction mixtures. The amount present in either phase was determined by comparison of peak height ratio with curves constructed from HPLC analysis of known amounts of each component dissolved in chloroform or in pH 4 acetate buffer (Fig. 1).

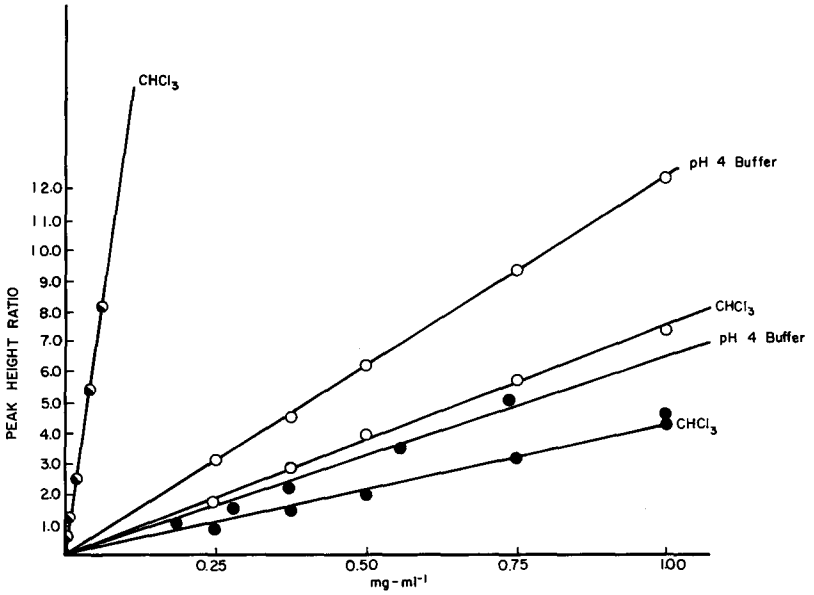

Fig. 1. Calibration curves for determining the extraction of efficiency of acetophenone oxime (◑), N-hydroxy-α-methylbenzylamine (●) and α-methylbenzylamine (○) from acetate buffer (pH 4) into chloroform.

Attempts were also made to extract the amine and hydroxylamine into chloroform over a pH range of 6.0–8.0, as an ion pair with heptafluorobutyric acid. This procedure, however, provided poorer extraction selectivity than extracting the components as the free base.

Derivatization

Derivatization of the hydroxylamine, amine and oxime was required prior to analysis. These components proved unstable to the stresses imposed by gas chromatographic analysis. The hydroxylamine disproportionates to oxime and amine. Instability of the amine and oxime was determined by the observation that GLC peak areas for the components were not linearly related to concentration. Furthermore, tailing of peaks was observed with non-derivatized molecules, necessitating their conversion to more chromatographable compounds.

The oxime, hydroxylamine and amine were converted to trimethylsilyl derivatives by reaction with BSA. This was the only silylating reagent (of the six tried) capable of reacting with all three compounds. Silylation of the oxime and hydroxylamine occurred easily and reaction could be completed within 5 min at room temperature. Among the silylating reagents tried [trimethylsilylchloride, BSA, hexamethyldisilazane, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylsilylimidazole and

tri-sil®], only BSA was capable of derivatizing the amine. Silylation of amine with this reagent, however, still required somewhat strenuous conditions. The reaction was allowed to proceed in dry acetonitrile with agitation at 60–70% for 30 min. Maximum yield of silylated products was obtained when the molar ratio of silylating reagent to analyte was 50–60:1. Under these conditions, 100% conversion of oxime, hydroxylamine and amine to the corresponding silyl derivative was observed, *i.e.* GLC analysis revealed no underivatized compound. When reaction time was shortened or the temperature lowered, less than quantitative silylation of amine was observed.

If provisions were made to exclude moisture from solutions containing these derivatives, they were stable for 48–72 h. At 72 h, only 3% loss of the hydroxylamine and 2% loss of oxime and amine were detected. All derivatives were stable for 24 h when no such precautions were taken.

Gas chromatographic analysis

The ketone and silylated derivatives of the amine, oxime and hydroxylamine were separated on a GP 5% SP-2401-DB-coated column with temperature programming. As shown in Fig. 2, this approach gives sharp well-defined peaks with good resolution of all components; while total analysis time for the four compounds was still only 7 min. The retention times for each of the components is shown in Table I. Quantitative analysis of the components was based on calibration curves generated for each compound. The amount was determined as a ratio of peak height of compound relative to N,N-dimethylaniline (retention time 4.5 min), present as internal standard. Standard curves were constructed with mixtures containing the four compounds of interest at thirteen different concentrations distributed over a concentration range from 0.3 to 500 μg/ml. All measurements were made in triplicate and plots of peak height ratio *vs.* concentration were constructed through linear regression analysis. Regression constants for all components are shown in Table I. Calibration curves were repeated on three consecutive days and were highly reproducible. Sensitivity limits for the method are *ca.* 0.3 μg/ml for each component (8 μg/ml for the amine) from 1-μl on-column injections. Precision between injections is $\pm 2\%$.

Determination of the optical composition of (R)- and (S)-α-methylbenzylamine mixtures

Resolution of optical mixtures of (*R*)- and (*S*)-forms of α-methylbenzylamine formed during enzymatic reduction of acetophenone oxime was accomplished by reaction of the enantiomeric amines with TPC[13] which converts them to diastereomeric amides separable by GLC on an OV-17 column. GLC analysis produced two peaks with retention times of 3.0 and 3.6 min (Fig. 3). The first peak corresponds to the amide formed from the (*R*)-amine, as determined by subsequent injection of an authentic sample of appropriately derivatized optically pure amine. This peak also contains small amount of the amide formed by reaction of the (*S*)-amine and (*R*)-acid chloride (present as a minor impurity in the resolving reagent), since (*R*,*S*)- and (*S*,*R*)-stereoisomers are, in fact, enantiomers and not resolvable. Quantitation of optical composition was made from peak ratio measurements. Percent optical composition was calculated making correction for the amount of (*R*)-TPC present as impurity in the resolving reagent using the equation[14]

$$R = \frac{50\,(R_{apL} - x)}{50 - x}$$

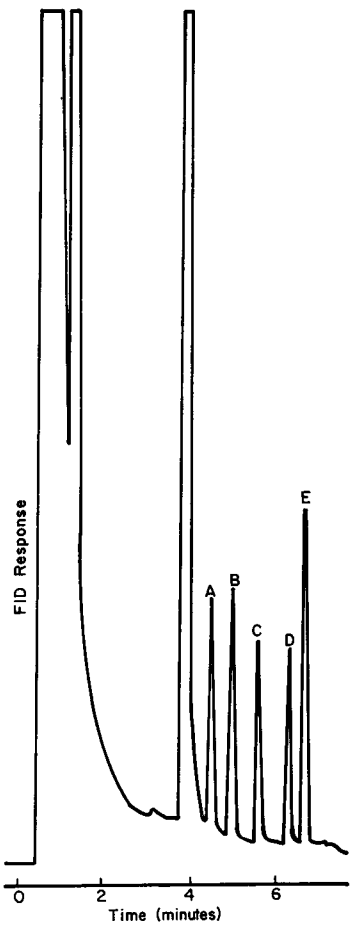

Fig. 2. Gas–liquid chromatogram of (A) N,N-dimethylaniline (internal standard), (B) silylated α-methylbenzylamine, (C) acetophenone, (D) silylated N-hydroxy-α-methylbenzylamine and (E) silylated acetophenone oxime, using the separation system described in the text.

where R is the calculated percentage of (R)-amine, R_{app} is the apparent percentage of (R)-amine determined from peak ratio measurements, and x is the percentage (R)-isomer in the TPC solution. Using mixtures of known optical composition, the percentage of (R)-amine in mixtures could be determined with an accuracy of $\pm 1\,\%$, over the complete enantiomeric composition range (*i.e.* 0–100 % (R)-amine in the mixture).

DISCUSSION

A rapid, simple and sensitive GLC analysis for acetophenone oxime and its major metabolites and breakdown product is described. Acetophenone oxime serves only as a model compound. Similar metabolic products have been isolated from both reductive oxime metabolism and oxidative metabolism of aliphatic primary amines (*e.g.* amphetamine). The method should serve as a general procedure for monitoring metabolic reactions involving such compounds.

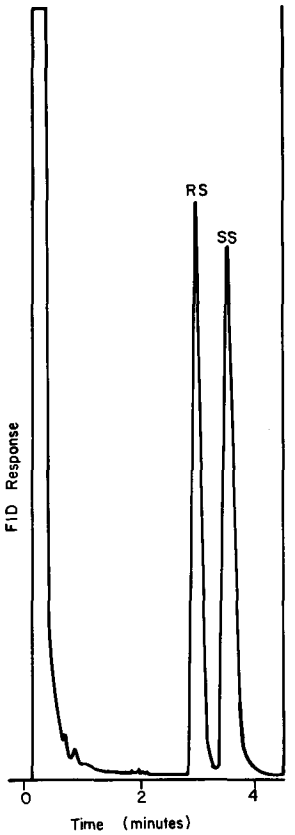

Fig. 3. Gas chromatogram showing the resolution of a racemic modification of (*RS*)-(±)-α-methylbenzylamine as diastereomeric trifluoroacetyl-(*S*)-prolyl amides.

Considerable care must be exercised in handling the aliphatic hydroxylamine. Beckett *et al.*[10] have recently shown the lability of such compounds at higher pH values and in the presence of many heavy metals. To preclude metal-catalyzed breakdown, EDTA or 8-hydroxyquinone must be added to the mixture. The hydroxylamine begins to show significant instability at pH > 7.5. Therefore, extraction must be carried out in two steps since the amine only exists to an appreciable extent as the free base above pH 10.5. The pH of the mixture was thus first adjusted to 4 to maximize hydroxylamine stability, where all components except the amine were extracted. At this pH, only 0.8 % of the hydroxylamine was lost in 4 h. Once the hydroxylamine had been extracted, the mixture could safely be made alkaline so that the amine could be extracted as its free base. This initial separation of amine from hydroxylamine was also necessary in instances where the optical composition of enantiomeric amine mixtures was to be determined, since both the amine and hydroxylamine are converted to the same amide after reaction with TPC.

Whereas the extraction efficiency of amine, oxime or ketone could be monitored spectrophotometrically, hydroxylamine extractability could only be monitored with its prior chromatographic separation. The hydroxylamine is readily oxidized under

numerous conditions to the oxime[10], which has a UV spectrum which overlaps with that of the hydroxylamine. The molar absorptivity of the oxime is, however, two orders of magnitude greater than that for the hydroxylamine; so that formation of a small amount of oxime would give exceedingly high results for the extraction efficiency of hydroxylamine determined by simple spectrophotometric analysis. Although the LC method used, clearly resolved all components, it is unacceptable for monitor-metabolic levels, because of the poor sensitivity of the method, when the effluent is monitored spectrophotometrically (UV), *i.e.* the molar absorptivity of amine and hydroxylamine are quite low ($\varepsilon \approx 200$ at 254 nm).

Quantitative derivatization of the oxime, amine and hydroxylamine was attained using BSA as silylating agent. The amine was resistant to derivatization and could only be silylated with this reagent and only under strenuous conditions. Silylation, however, enhanced the stability and reduced tailing allowing the components to be rapidly analyzed at submicrogram levels. The standard error of regression given in Table I indicate the goodness of fit of the regression lines generated from 39 data points at 13 different concentrations for each analyte. Derivatization of the hydroxylamine has alternatively been reported using BSTFA[9,15] or trifluoroacetic anhydride[16]. However, the other methods do not provide a none-step analysis of all four components of interest. BSTFA, furthermore, did not effectively silylate the amine and was therefore not a suitable reagent.

Reductive metabolism of oximes proceeds with the generation of an asymmetric center. To determine the optical composition of the enantiomeric amine mixture formed during microsomal oxime reduction, the amines were converted to diastereomeric amides with a chiral acid chloride; the amides were then separated by GLC. The (*R*)-isomer eluted prior to the (*S*)-amine. This is the same order of elution found by Gordis[13] for amphetamine reacted with (*S*)-TPC and by Martin *et al.*[11] who reacted various β-phenylisopropyl amines with N-pentafluorobenzoyl-(*S*)-(—)-prolyl-l-imidazole to effect GLC resolution.

Attempts to resolve the enantiomeric N-hydroxy-α-methylbenzylamines by reaction with (*S*)-TPC to form diastereomeric hydroxamic acids were unsuccessful. GLC of the "derivatized" mixtures indicated that the hydroxylamines are converted to the same products which form on reaction with α-methylbenzylamine. This reaction has not been investigated, but since it may proceed with loss of chirality, it is inappropriate for analysis of the hydroxylamines. Since the hydroxylamine and amine are quantitatively separated by extraction prior to derivatization, the optical composition of the amine can still be determined without interference from the hydroxylamine.

Although other methods are available for analysis of such mixtures, they(1) require multiple-column analysis, (2) require long analysis times, and (3) lack the precision and simplicity of this method.

ACKNOWLEDGEMENT

This work was supported in part by NIH grant CA-18615 provided by the National Cancer Institute (PHS DHEW).

REFERENCES

1 A. H. Beckett, J. M. Van Ryk, H. M. Chissick and J. W. Gorrod, *J. Pharm. Pharmacol.*, 23 (1971) 560.
2 A. H. Beckett and S. Al-Sarraj, *J. Pharm. Pharmacol.*, 24 (1972) 174.
3 K. K. Midha, A. H. Beckett and A. Saunders, *Xenobiotica*, 4 (1974) 627.
4 H. B. Hucker, B. M. Michniewicz and R. E. Rhodes, *Biochem. Pharmacol.*, 20 (1971) 2123.
5 C. J. Parli, N. Wang and R. E. McMahon, *J. Biol. Chem.*, 246 (1971) 6953.
6 C. J. Parli and R. E. McMahon, *Drug Metab. Disp.*, 1 (1973) 337.
7 J. Hes and L. A. Sternson, *Drug. Metab. Disp.*, 2 (1974) 345.
8 L. A. Sternson and J. Hes, *Pharmacology*, 13 (1975) 234.
9 A. H. Beckett and K. Haya, *J. Pharm. Pharmacol.*, 29 (1977) 89.
10 A. H. Beckett, A. R. Purkaystha and P. H. Morgan, *J. Pharm. Pharmacol.*, 29 (1977) 15.
11 S. B. Martin, M. Rowland and N. Castagnoli, *J. Pharm. Sci.*, 62 (1973) 821.
12 H. Feuer, B. F. Vincent and R. S. Bartlett, *J. Org. Chem.*, 30 (1965) 2877.
13 E. Gordis, *Biochem. Pharmacol.*, 15 (1966) 2124.
14 *TPC product literature*, Regis, Chicago, Ill.
15 B. Lindeke, A. K. Cho, T. L. Thomas and L. Michaelson, *Acta Pharm. Suecica*, 10 (1973) 493.
16 J. Wright, A. K. Cho and J. Gal, *Life Sci.*, 20 (1977) 467.