Journal of Chromatography, 188 (1980) 159–167
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,362

CONVERSION OF ARYLHYDROXYLAMINES TO ELECTROCHEMICALLY-ACTIVE DERIVATIVES SUITABLE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS WITH AMPEROMETRIC DETECTION

DONALD G. MUSSON and LARRY A. STERNSON*

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kan. 66044 (U.S.A.)

(First received May 14th, 1979; revised manuscript received August 29th, 1979)

SUMMARY

A sensitive method is described for the analysis of arylhydroxylamines (derived metabolically from arylamines and nitro compounds) in liver homogenates. The procedure is based on initial extraction of the analyte from the biological matrix with dichloromethane; derivatization in the extraction solvent with *p*-dimethylaminophenylisocyanate to form a hydroxyurea which is subsequently separated from co-extracted materials and reaction by-products by high-performance liquid chromatography (HPLC). The hydroxylamine, itself, is quite unstable [$t_{1/2} = 8$ min in pH 7.4 phosphate buffer (0.1 M; $\mu = 0.5$)]; conversion to the hydroxyurea increases its stability by a factor of 500. The hydroxyurea derivative strongly absorbs ultraviolet light ($\lambda_{max.} = 268$ nm; $a_m = 37,200$) and is also electrochemically oxidized ($E_p = +0.38$ V *vs.* Ag/AgCl) at a glassy carbon surface. The HPLC eluent is therefore monitored both spectrophotometrically (at 254 nm) and amperometrically (at 0.50 V at a glassy carbon anode). Optical detection offers a sensitivity of $9 \cdot 10^{-7}$ M while electrochemical detection affords a sensitivity limit of $1 \cdot 10^{-8}$ M.

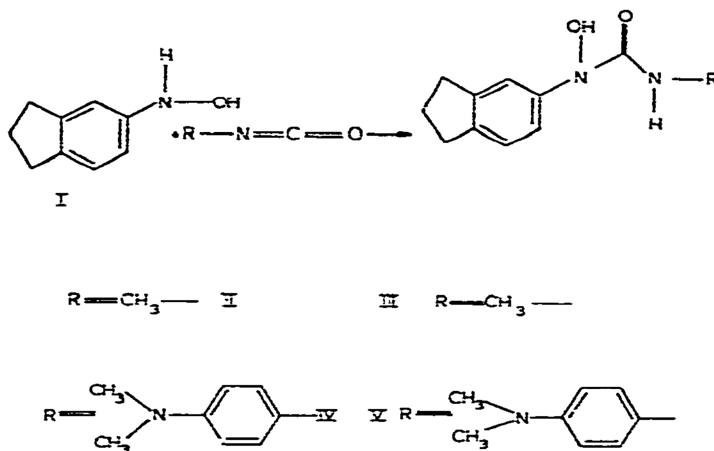
INTRODUCTION

The carcinogenicity of certain aromatic amines and nitro compounds is related to their metabolic conversion in various tissues to N-hydroxylamines[1,2]. Available analytical methods for quantitification of arylhydroxylamines in biological media[3–9] are often inadequate in that they either lack sufficient sensitivity, lack specificity and/or fail to take into consideration the instability of the analyte in aqueous solution. Arylhydroxylamines have been separated from other amine (nitro) metabolites by high-performance liquid chromatography (HPLC) with spectrophotometric[10] or amperometric[11] monitoring of analytes. Although these procedures provided specificity they lacked sufficient sensitivity (concentrations $\leqslant 5 \cdot 10^{-6}$ M) due in part to on-column degradation of the analyte. More recently, a method has been described[12]

* To whom correspondence should be addressed.

in which arylhydroxylamines (I) extracted from biological fluid into ether, are reacted with methylisocyanate (II) and the resulting hydroxyurea (III) chromatographed on a reversed-phase column with spectrophotometric detection of the effluent. This procedure stabilizes the analyte through derivatization and provides specificity through HPLC separation. However, due to the low molar absorptivity of the hydroxyurea the method is somewhat insensitive (responds to concentrations of I $\geqslant 2 \cdot 10^{-6}$ M).

In this report we describe a modification of this derivatization procedure in which the extracted hydroxylamine is reacted with *p*-dimethylaminophenylisocyanate (IV) to form a hydroxyurea (V) which is readily oxidized at carbon electrodes[13]. The derivative is separated from co-extracted materials by HPLC and detected in the effluent by either spectrophotometry or amperometry, to provide a means for quantifying trace levels of arylhydroxylamines specifically and as stable products.



EXPERIMENTAL

Apparatus

Chromatography was performed using a Waters Assoc. Model 6000 A solvent delivery system, U6K septumless injector, a Model 440 absorbance detector operated at 254 nm (Waters Assoc., Milford, Mass., U.S.A.) and a Brinkmann (Westbury, N.Y., U.S.A.) Model EA-1096 electrochemical detector cell consisting of two miniature glassy carbon surfaces serving as working and auxiliary electrodes, and a Ag/AgCl rod immersed in 3 M KCl solution as reference electrode. Potentials were controlled with an apparatus that was laboratory built as described by Kissinger *et al.*[14].

Materials

Dichloromethane (HPLC grade), methanol (HPLC grade) and triethanolamine were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and were used without further purification. *p*-N,N-Dimethylaminoaniline·2 HCl was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.) and purified by recrystallization from absolute ethanol with decolorizing carbon (m.p. 204–206°).

5-Hydroxyaminoindan (I) was synthesized by reduction of 5-nitroindan (Aldrich, Milwaukee, Wisc., U.S.A.) with zinc and ammonium chloride[15] and characterized from melting point, infrared (IR), nuclear magnetic resonance spectrometry (NMR) and mass spectral data[12]. 5-Nitrosoindan was prepared as described by Mijs *et al.*[16]. 5,5'-Azoxyindan was synthesized by H_2O_2 oxidation of I (ref. 17).

4-Dimethylaminophenyl isocyanate (IV) was synthesized by mixing 4-dimethylaminoaniline (0.65 g, 4.8 mmol) with a 20% solution of phosgene in toluene (6 ml). The solution was stirred at 110° for 30 min. The toluene was then removed under reduced pressure yielding a pinkish white solid; the hydrochloride salt of IV in 84% yield (m.p. 241–242°). The free base, IV, was liberated by slowly adding its hydrochloride salt (3.88 g, 23.9 mmol) to a stirred mixture of dichloromethane–water (1:1, 300 ml) containing 3 g of potassium carbonate. The dichloromethane layer was removed, concentrated on a rotatory evaporator and the residue was sublimed at 2 mm, 90–110° giving IV in 40% yield as a white crystalline material. The identity of the product (m.p. 34–35°) was confirmed by IR spectra (in $CHCl_3$, 2250 cm^{-1} N=C=O stretch), electron-impact mass spectrometry ((EI-MS); 33°, molecular ion at *m/e* 162) and elemental analysis, calculated for $C_9H_{10}N_2O$: C, 66.67, H, 6.17; N, 17.28. Found: C, 66.27; H, 6.38; N, 17.52.

A material also crystallized from the organic layer during liberation of the free base of the isocyanate. This material had a crude melting point of 238–240°; crystallization from 100% ethanol gave a production m.p. of 255–260°. EI-MS of this product gave a molecular ion at *m/e* 298 suggesting that the compound was 1,3-bis-(4'-dimethylaminophenyl)urea (VI). Structural identity was confirmed by elemental analysis calculated for $C_{17}H_{22}N_4O$; C, 68.45; H, 7.38; N, 18.79. Found: C, 68.10; H, 7.65; N, 18.52, and from an IR spectrum ($CHCl_3$) which gave a prominent band at 1675 cm^{-1} (C=O stretch).

1-Hydroxy-1-(5'-indanyl)-3-(*p*-dimethylaminophenyl)urea (V) was synthesized by mixing I (0.19 mol) with IV (0.20 mol) in dichloromethane. The solution was then cooled in wet ice; compound V precipitated in 78% yield and was crystallized from benzene giving a product with a melting point of 143–145°. Mass spectra gave a molecular ion at *m/e* 311 consistent with V. IR spectroscopy only gave a strong band at 1675 cm^{-1} (C=O stretch, $CHCl_3$). Elemental analysis confirmed the identity of the product calculated for $C_{18}H_{21}N_3O_2$: C, 69.45; H, 6.75; N, 13.50. Found: C, 69.48; H, 6.93; N, 13.24.

Liver homogenates were prepared as previously described[19]. The supernatants remaining after centrifugation of crude homogenates at 9000 *g* (100 mg of liver/0.5 ml of buffer) were diluted 10-fold and used as such in all experiments.

Procedures

Extraction. Samples of I (in concentrations ranging from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-8}$ *M*) were dissolved in either 5 ml of Tris–HCl buffer (0.02 *M*, pH 7.4) or in 5 ml of liver homogenate. The samples were extracted with 8 ml (1.5 volumes) of dichloromethane by vortexing the mixture in 15 ml, conical centrifuge tubes for 3 min and then centrifuging the tubes for 20 min at 10,000 *g* to separate phases. The upper aqueous layer was removed by aspiration and disgraded.

Derivatization. The dichloromethane extracts were maintained at 0° before derivatization to supress oxidation of I in water-saturated dichloromethane. Com-

pound IV (150 µl of a solution containing 15 mg/10 ml dichloromethane) was added to the extracts, mixed for 1 min and then, three drops (by Pasteur pipette) of triethanolamine was added, mixed and the solution extracted with phosphate buffer (5 ml. 0.05 M; pH 10). The aqueous layer was removed by aspiration and the dichloromethane was then evaporated to dryness in a stream of nitrogen. The residue was reconstituted in 0.25 ml of dichloromethane or methanol. These samples were then maintained at 0° until analyzed.

Chromatography. Samples were analyzed by HPLC on a µBondapak C_{18} column (300 cm × 4 mm I.D.; Waters Assoc.) operated at a flow-rate of 1.5 or 2.0 ml/min with methanol–water–acetic acid (33:49:1) as mobile phase. Column effluent was passed initially through a spectrophotometric detector operated at 254 nm and then through an amperometric detector (consisting of glassy carbon working and auxiliary electrodes and a Ag/AgCl reference electrode) maintained at a potential of +0.5 V (*vs.* Ag/AgCl). Ten-microliter injections were made on-column from all samples. For quantitative analysis, concentrations of I were determined from standard curves prepared by analysis of samples of liver homogenates (or buffer) containing known amounts of N-hydroxylaminoindan. Calibration curves were constructed by plotting peak area *vs.* analyte concentration for eight concentrations of I in the range from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-5}$ M. All analysis were made in triplicate, peak areas measured by planimetry and data subjected to linear regression analysis.

RESULTS

Analysis of arylhydroxylamines in liver homogenates is carried out in three stages: (1) separation of analyte, I, from the biological matrix by solvent extraction, (2) conversion of I to V in the extraction solvent and (3) separation of V from co-extracted materials by HPLC with direct spectrophotometric and amperometric monitoring of the column effluent.

Extraction

Extraction of N-hydroxylamines from aqueous solution was accomplished with dichloromethane as previously described and afforded 88 ± 5% recovery of I from buffer solutions and 82 ± 6% extraction from liver microsomal suspensions over the concentration range $1 \cdot 10^{-7}$–$1 \cdot 10^{-5}$ M.

Derivatization

A structurally modified isocyanate was sought which would form a stable product with I, and would contain functionalities permitting its detection at very low levels.

Amperometric detection of HPLC column effluent offers a sensitive means of monitoring trace levels of analytes that respond electrochemically[19]. Response is favored with molecules that are electrochemically transformed at low potentials ($E_p \leq 0.6$ V). Accordingly, a suitable reagent was sought that would yield an electroactive product upon reaction with arylhydroxylamines. *p*-N,N-Dimethylaminoaniline is readily and reversibly oxidized at solid anodes[12] ($E_{p/2} = +0.31$ V at glassy carbon anode *vs.* Ag/AgCl). Condensation of this substituted aniline with phosgene affords the corresponding isocyanate (IV) which is electrochemically inactive (at anodic

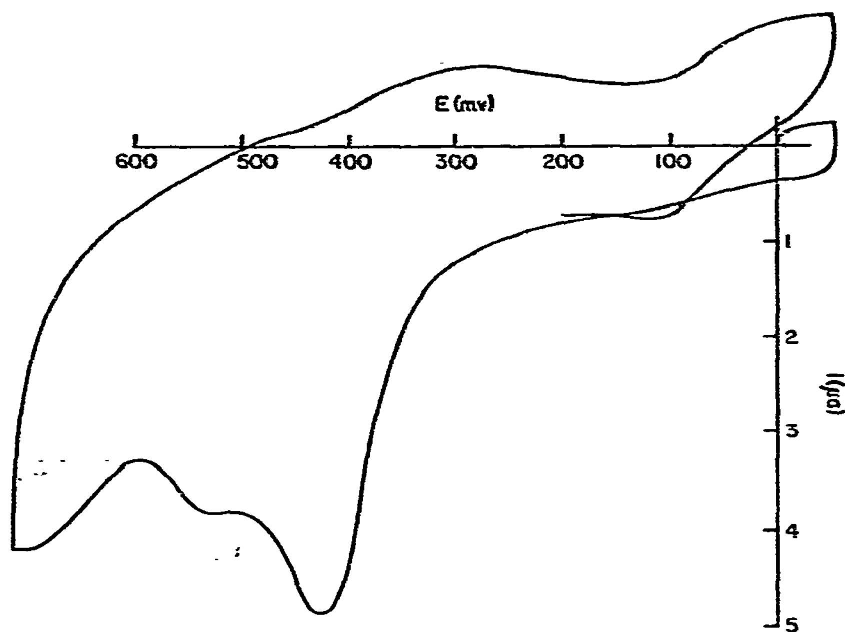
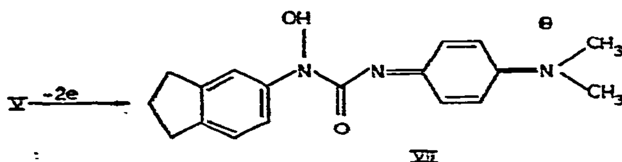


Fig. 1. Cyclic voltammogram demonstrating the oxidation of 1-hydroxy-1-(5'-indanyl)-3-(*p*-dimethylaminophenyl)urea (V) in HPLC mobile phase [methanol–water–acetic acid (33:49:1)] at a glassy carbon electrode (*vs.* a Ag/AgCl reference electrode). Scan rate: 9 V/min.

potentials); however upon reaction of IV with I, a hydroxyurea (V) is formed which as shown by cyclic voltammetry (Fig. 1) is readily and irreversibly oxidized ($E_{p/2}$ = +0.38 V *vs.* Ag/AgCl) at a glassy carbon electrode in an unstirred hydroalcoholic solution. This oxidation has been shown coulometrically to be a two electron process, apparently involving formation of VII.



This isocyanate (IV), is the first example of an electrogenic reagent (by analogy with fluorogenic and chromogenic reagents) for HPLC, being itself electrochemically inactive, but forming electro-oxidizable products upon reaction with arylhydroxylamines. The electrochemical reaction was shown to be suitable for monitoring V in HPLC column effluents in that in a flowing stream, peak current (i_p) measured at +0.5 V was linearly related to analyte (V) concentration over the range $5 \cdot 10^{-8}$–$1 \cdot 10^{-6}$ *M*.

Derivatization of I with IV was carried out directly in the extraction solvent, dichloromethane, using an approximate 200-fold excess of reagent. The product formed during this reaction, 1-hydroxy-1-(5'-indanyl)-3-(*p*-dimethylaminophenyl)urea (V) was characterized from mass spectra (Fig. 2), IR and NMR spectra and elemental

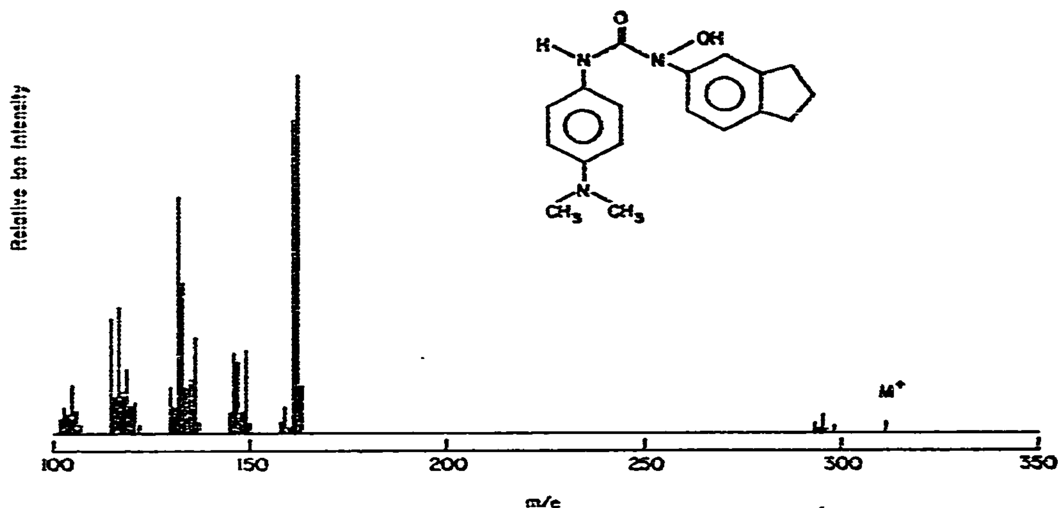
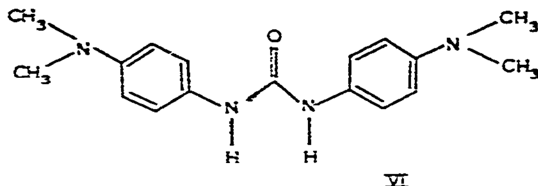


Fig. 2. Electron-impact mass spectrum of 1-hydroxy-1-(5'-indanyl)-3-(*p*-dimethylaminophenyl)urea (V) recorded at 70 eV.

analysis. Under the pseudo-first order reaction conditions chosen for derivatization, I. was quantitatively converted to V in 1 min at room temperature (20–23°).

A major by-product formed during this reaction was isolated and subsequently identified as 1,3-bis-(*p*-dimethylaminophenyl)urea, VI. It forms primarily as a result of hydrolysis of IV



yielding *p*-dimethylaminoaniline which then condenses with a second mole of IV. The extent of this reaction can be reduced significantly be eliminating water from the solvent after extraction and prior to the addition of IV. The urea, VI, is also formed by direct reaction of V with *p*-dimethylaminoaniline, although this reaction proceeds slowly.

The excess isocyanate also catalyzes the degradation of the hydroxyurea (V) through apparent O-acylation to yield three products of unknown composition (as observed by HPLC). The excess isocyanate can, however, be conveniently removed, and this stability problem thus circumvented by addition of an excess of triethanolamine to the reaction mixture after derivatization is deemed complete. The isocyanate is thus instantaneously (reaction is complete in <1 min) and quantitatively converted to a water soluble carbamate that partitions favorably into aqueous buffer (pH 10). Removal of IV by this procedure minimized degradation of V and subsequently provides simple chromatograms (HPLC) with no interference observed to the analyte

(V) peak when monitored either spectrophotometrically (at 254 nm) or amperometrically (at +0.50 V *vs.* Ag/AgCl electrode). Using this procedure, the hydroxyurea(V), obtained from derivatization of liver microsomal extracts, is stable for 14 h at 0°; at room temperature, degradation is significantly accelerated. Accordingly, samples are maintained $\leqslant 0°$ prior to chromatographic analysis.

Chromatography

The dichloromethane solutions are evaporated to dryness and the residues reconstituted with 250 μl of this solvent, concentrating the sample and thus improving the ultimate sensitivity of the method. The derivatized product is separated from co-extracted and reaction by-products by reverse phase partition chromatography with isocratic elution of components with methanol–water–acetic acid (33:49:1) mobile phase (Fig. 3). The peak corresponding to V is well resolved (retention volume $V_r = 19.8$ ml) from other products of amine metabolism: derivatized and underivatized 5-aminoindan ($V_r = 4.1$ and 10.1 ml, respectively), 5-nitrosoindan ($V_r = 84$ ml), 5-nitroindan ($V_r = 83.2$ ml) and 5,5'-azoxyindan ($V_r > 300$ ml); and is also well separated from the peak rising from the symmetrical urea, VI ($V_r = 6.2$ ml) and other extraneous peaks.

The hydroxylamine, I, is quantitated as the hydroxyurea, V by comparing peak area with a standard curve constructed from data derived from analysis of liver microsomal suspensions containing known amounts of I (at six different concentrations equally spaced over the indicated range). Column effluent was passed sequentially through spectrophotometric and amperometric detectors. A linear relationship was found between the concentration of I and peak area over the range from $5 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$ *M* by spectrophotometry and from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ *M* by amperometry. Linear regression analysis of this data generated the lines, peak area = 0.010 (I) with a correlation coefficient of 0.992 for ultraviolet detection and the line, peak area = 0.012 (I)–0.033 with a correlation coefficient of 0.995 for electrochemical detection. Total analysis with spectrophotometric detection was carried out with reproducibility of ± 6, and $\pm 7\%$ with electrometric detection. Accuracy of determinations based on the amount of I determined as V after extraction from microsomal suspensions, derivatization and HPLC was $\pm 5\%$ as determined from HPLC analysis of known amounts of V. The minimum concentration of I present in liver suspensions which could be determined with this precision and accuracy was $9.4 \cdot 10^{-7}$ *M* by spectrophotometry and $3 \cdot 10^{-8}$ *M* by amperometry, from 10-μl injection volumes onto the column.

DISCUSSION

A new reagent for use in the HPLC analysis of arylhydroxylamines is described. Conversion of the hydroxylamine to a hydroxyurea stabilizes I, and yields a derivative which can be selectively chromatographed and detected at 100-times lower levels than achievable by previous methods[10–12]. An additional advantage of this reagent (IV) is that it is not itself electroactive but reacts with nucleophiles to form products which are electro-oxidized at glassy carbon electrodes at low potentials. Because few compounds are oxidized at these potentials a large signal-to-noise ratio is observed and high sensitivity achieved.

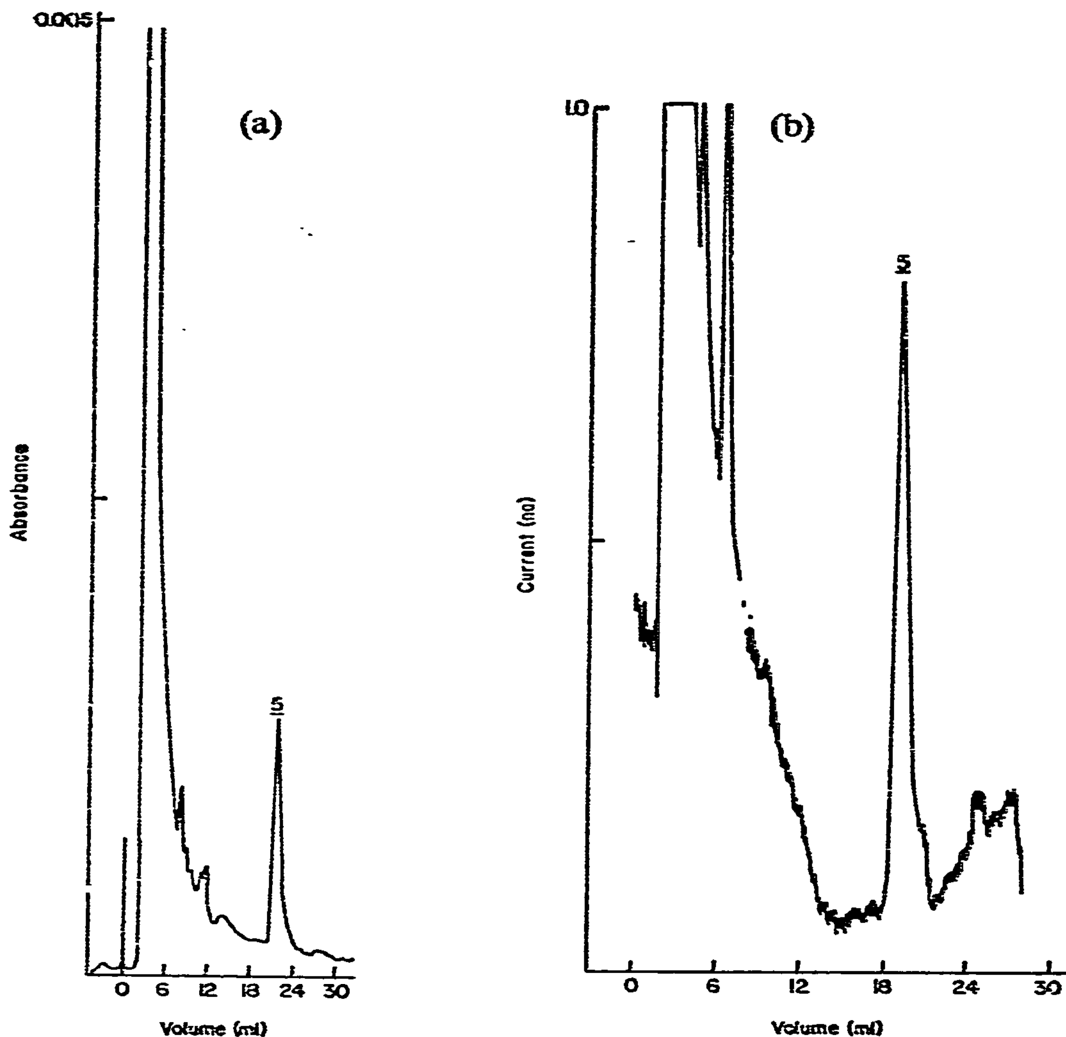


Fig. 3. Chromatogram of 1-hydroxy-1-(5'-indanyl)-3-(*p*-dimethylaminophenyl)urea (V) obtained by reaction of hydroxyaminoindan extracted from liver microsomal homogenates with *p*-dimethylaminophenylisocyanate (IV). Separation carried out on an RP-18 column with methanol–water–acetic acid (33:49:1) mobile phase and flow-rate = 1.5 ml/min Analyte detected (a) spectrophotometrically at 254 nm and (b) amperometrically at +0.5 V *vs* Ag/AgCl reference electrode

The reaction between arylhydroxylamine (I) and IV is quantitative and rapid under the conditions chosen. Excess reagent must, however, be removed soon after reaction is complete because it interferes with the ensuing analysis. In previous studies with methyl isocyanate[12], excess reagent could be removed by evaporation under a stream of nitrogen because of its high vapor pressure (b.p. 38°); IV, however, is non-volatile. Destruction of excess reagent (IV) with water or aqueous acid or base was unsuitable because it produced large quantities of the symmetrical urea, VI, which subsequently gave poor chromatograms. The use of triethanolamine offers a

convenient means of removing excess reagent in that it reacts quantitatively and very rapidly with IV (but does not react with V under the conditions of the reaction) to yield a product which partitions favorably into pH 10 aqueous buffer solution (V does not extract into this buffer). In this way, all problems associated with the use of IV for analysis of arylhydroxylamines are avoided and the method is reliable, durable and offers high sensitivity.

ACKNOWLEDGEMENTS

The work was supported in part by NIH grants CA-09242 and CA-19288 from the National Cancer Institute and a grant from the General Research Fund of the University of Kansas (Lawrence, Kan., U.S.A.).

REFERENCES

1 E. C. Miller and J. A. Miller, *Pharmacol. Rev.*, 18 (1966) 805.
2 J. A. Miller, J. W. Cramer and E. C. Miller, *Cancer Res.*, 20 (1960) 950.
3 F. Herr and N. Kiese, *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol.*, 235 (1959) 351.
4 E. Bamberger, *Justus Liebigs Ann. Chem.* 424 (1925) 233.
5 R. E. Gammans, J. T. Stewart and L. A. Sternson, *Anal. Chem.*, 46 (1974) 620.
6 A. C. Bratton, E. K. Marshall, D. Babbit and A. R. Hendrickson, *J, Biol. Chem.*, 128 (1939) 537.
7 L. A. Sternson and J. T. Stewart, *Anal. Lett.*, 6 (1973) 1055.
8 L. A. Sternson, *Anal. Chem.*, 46 (1974) 2228.
9 H. Gans, C. S. Lieber and R. Rabin, *J. Pharmacol. Exp. Ther.*, 183 (1972) 218.
10 L. A. Sternson and W. J. DeWitte, *J. Chromatogr.*, 137 (1977) 305.
11 L. A. Sternson and W. J. DeWitte, *J. Chromatogr.*, 138 (1977) 229.
12 L. A. Sternson, W. J. DeWitte and J. G. Stevens, *J. Chromatogr.*, 153 (1978) 481.
13 D. W. Fassett, D. B. Glass, T. H. James, D. B. Julian, W. R. Ruby, J. M. Swell, J. H. Sterner, J R Thintle, P W. Vittum and A. Weissburger, *J. Amer. Chem. Soc.*, 73 (1951) 3100.
14 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
15 E. E. Smissman and M. D. Corbett, *J. Org. Chem.*, 37 (1972) 1847.
16 W. J. Mijs, S. E. Hoekstra, R. M. Ulmann and E. Havinga, *Rec. Trav. Chem.*, 77 (1958) 746.
17 R. M. Peck and H. J. Creech, *J. Amer. Chem. Soc.*, 74 (1952) 468.
18 R. A. Wiley, L. A. Sternson, H. A. Sasame and J. R. Gillette, *Biochem. Pharmacol.*, 21 (1972) 3235
19 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 447A.