Journal of Chromatography, 153 (1978) 481–488

CHROM. 10,749

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF ARYLHYDROXYLAMINES AFTER DERIVATIZATION WITH METHYL ISOCYANATE

LARRY A. STERNSON*, WAYNE J. DeWITTE and JANICE G. STEVENS

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

(First received October 21st, 1977; revised manuscript received November 15th, 1977)

SUMMARY

The quantitative analysis of micromolar levels of arylhydroxylamines in liver homogenates is described. The analyte is extracted from the biological medium with dichloromethane and then converted to the corresponding N-hydroxyurea by reaction with methyl isocyanate. The reaction product is stable both in the dry state and in solution. The derivatized mixture is separated by reversed-phase partition chromatography and the hydroxylamine quantitated as the methylhydroxyurea by spectrophotometric monitoring of the column eluent at 254 nm. The method is specific for arylhydroxylamines in that the product is different from products formed by reaction of methyl isocyanate with alternate amine metabolites and metabonates.

INTRODUCTION

Some primary aromatic amines and amides are thought to induce cancer through interaction of their N-hydroxylated metabolites with nucleic acids and protein[1,2]. The low concentrations at which these compounds are present in biological samples and their ease of oxidation have hindered development of reliable methods for their analysis, particularly in the presence of other metabolites. Most reported methods lack sufficient specificity to differentiate between the arylhydroxylamines and C-hydroxylated metabolites of aryl amines. Furthermore, reported procedures often measure arylhydroxylamines by conversion to nitroso[3] or azo[4] derivatives which are indistinguishable from alternate metabolic products, formed via different metabolic routes. Ultimate detection of these analytes has involved spectrophotometry[5–7], fluorimetry[8], amperometry[9] and isotopic measurements[10].

We have recently described a high-pressure liquid chromatographic (HPLC) separation and quantitation of C- and N-hydroxylated metabolites and metabonates of the aromatic amine, aniline, using reversed-phase partition chromatography with ultraviolet spectrophotometric[11] or amperometric[12] detection of analytes. These

* To whom correspondence should be addressed.

methods are specific, sensitive (detection limit: 10^{-8} *M*) and reliable for analysis of C-hydroxylated metabolites of arylamines. However, although these procedures can specifically monitor N-hydroxylamines, the instability of these compounds limits the concentration that can be quantitated to $5 \cdot 10^{-5}$ *M* and demands rapid processing and analysis of samples. It is not possible to practically monitor arylhydroxylamines in biological fluids with these methods, because of their short half lives, without stabilization of the analyte early into the analysis scheme. In this report we describe a derivatization procedure for arylhydroxylamines prior to their HPLC separation, which renders the analyte stable in solution and converts the hydroxylamine to a species which can be distinguished from all other amine metabolites and metabonates.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Model M 6000A solvent delivery system, a Model U6K septumless injector and Model 440 absorbance detector operated at 254 nm (Waters Assoc., Milford, Mass., U.S.A.).

Reagents

Methanol was ChromAR grade (Mallinckrodt, St. Louis, Mo., U.S.A.). Distilled water was used throughout. Analytical-reagent grade dichloromethane (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was used without further purification. Methyl isocyanate was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was used as received.

5-Hydroxyaminoindan (HAI) was synthesized by reduction of 5-nitroindan (Aldrich) with zinc and ammonium chloride[13]. The product was isolated as yellowish-white plates with a melting range of 64–66°. Spectrometric data, including ultraviolet and mass spectra, and elemental analysis confirmed the identity of the product (elemental analysis: calculated, C, 72.44%; H, 7.44%; N, 9.39%; found, C, 73.83%; H, 7.50%; N, 9.50%).

1-Hydroxy-1-(5'-indanyl)-3-methylurea (HIMU) was synthesized by established methods[14], mixing a solution of HAI in diethyl ether with a 25 molar excess of an ether solution of methyl isocyanate. The product, after recrystallization from benzene, was white and crystalline with a melting point of 119.5–120.0°. Spectrometric data and elemental analysis confirmed the identity of the product (elemental analysis: calculated, C, 57.81%; H, 6.08%; N, 16.86%; found, C, 58.01%; H, 5.95%; N, 16.98%). Mass spectra gave a peak for the molecular ion at *m/e* 206, and a fragmentation pattern consistent with the behavior of similar substituted hydroxyureas[15].

Liver homogenates

Male Sprague-Dawley rats (weighing 200–250 g) were decapitated, their livers excised and washed in cold 0.02 *M* Tris-HCl buffer (pH 7.4). The liver was homogenized with a PTFE pestle in four volumes of the buffer. The homogenate was centrifuged at 9000 *g* for 20 min at 4°. The supernatant was removed, diluted 10-fold with buffer and used as such in all experiments.

PROCEDURES

Extraction

Varying known amounts of HAI were added to either 5 ml of Tris-HCl buffer (0.02 *M*; pH 7.4) or 5 ml of liver homogenate to give final solutions containing $1 \cdot 10^{-4}$ to $1 \cdot 10^{-6}$ *M* HAI. The mixtures were extracted for 5 min with 8 ml of dichloromethane, and then centrifuged at 800 *g* for 5 min. The aqueous layer and protein precipitate at the solvent interface were aspirated to waste.

Derivatization

Methyl isocyanate (400 μl of undiluted material) was added to a 3-ml aliquot of the dichloromethane extract and the solution was shaken for 1 min at room temperature. The solvent was then evaporated to dryness in a nitrogen atmosphere and the residue redissolved in 250 μl of methanol–water (50:50) containing $4.73 \cdot 10^{-5}$ *M* N,N-dimethylaniline (present as the internal standard).

Chromatography

Samples were analyzed by HPLC using a μBondapak C_{18} column (30 cm $\times$ 4 mm I.D.; Waters Assoc.) operating at a flow-rate of 2 ml/min (2300 p.s.i.) with methanol–water (50:50) as mobile phase. Analytes were detected spectrophotometrically at 254 nm. For quantitative analysis concentrations of hydroxylamine were determined from a standard curve. A stock solution of HIMU ($1 \cdot 10^{-3}$ *M*) was prepared in methanol–water (50:50) and diluted as required with the same solvent. N,N-Dimethylaniline (final concentration $4.73 \cdot 10^{-5}$ *M*) was added to each solution as internal standard. All quantitative measurements were made relative to this constant amount of dimethylaniline. Standard curves were constructed by plotting peak height ratio (analyte: internal standard) *vs.* analyte concentration for 6 concentrations of HIMU in the range from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*. All measurements were made in triplicate and the data subjected to linear regression analysis.

RESULTS

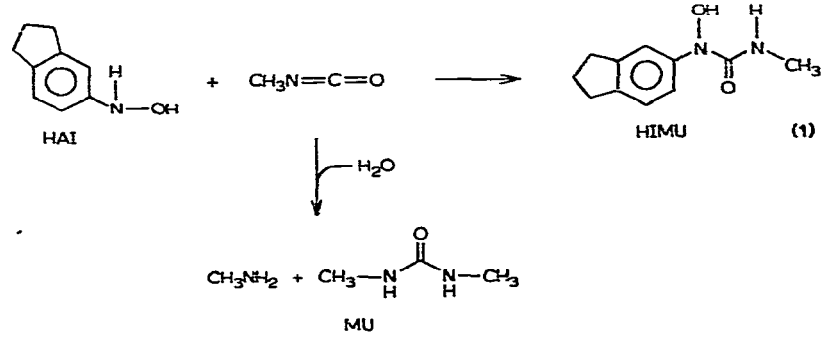
Analysis of the HAI in liver homogenates was performed in three stages: (1) initial extraction of the analyte; (2) subsequent derivatization by reaction with methyl isocyanate; and (3) separation of the derivatized mixture by high-performance reversed-phase partition chromatography.

Extraction

The arylhydroxylamine was removed from aqueous buffer or liver homogenate solutions by extraction with 1.6 volumes of dichloromethane. For biological samples 5 min extractions were required to reach equilibrium. One extraction removed 88 $\pm$ 2% of the analyte from aqueous solutions, and 82% from tissue homogenates, over the concentration range ($1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*) studied. Extraction could alternatively be carried out with chloroform, however, this solvent was unsatisfactory for subsequent derivatization. Centrifugation of the biological fluid-dichloromethane system was required after agitation to form two distinct liquid phases, thereby maximizing analyte recovery.

Derivatization

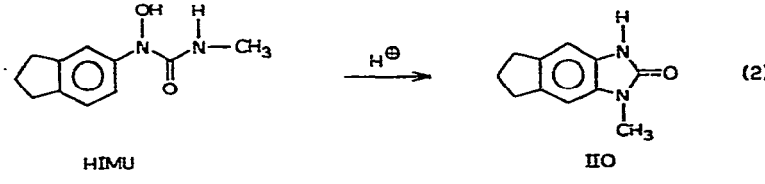
The extracted hydroxylamine was reacted with methyl isocyanate. The reaction product was isolated by HPLC and identified from elemental analysis and mass spectra as the N-substituted monoacylated hydroxylamine (HIMU) (eqn. 1)



For analysis from biological fluids the reaction was carried out in the presence of a 1000-fold excess of isocyanate. This large excess of reagent was required because isocyanates react indiscriminately with nucleophilic species and therefore are consumed by compounds which may co-extract (*e.g.* water, alcohols, amines) with HAI into dichloromethane. Sufficient reagent must be present to totally derivatize the analyte in the presence of these potential interferents. Acylation reactions are, therefore, carried out under anhydrous conditions. In this regard, the derivatizing agent also acts as a dessicant reacting with water to form methyl urea (MU) (eqn. 1). For the convenience of the analyst, reaction was carried out at room temperature in the untreated dichloromethane extract. Under these conditions reaction was complete in less than 1 min and HAI was quantitatively converted to the corresponding hydroxyurea, HIMU.

HIMU was stable at room temperature in the extraction solvent or HPLC mobile phase (methanol–water (50:50)) for more than 1 h, *i.e.* less than 5% loss was observed over this time period. The derivative appears to be indefinitely stable (more than 6 weeks) when stored dry (*i.e.* in the absence of solvent) at room temperature. Reaction must, however, be carried out in the absence of acid to prevent the acid catalyzed conversion of HIMU to the corresponding benzimidazol-2-one (IIO) (eqn. 2)[15].

Chloroform was unsuitable as the reaction medium, because of the relatively high concentration of ethanol which is added to stabilize the commercially available solvent. Ethanol consumes the reagent, converting it to N-methyl-O-ethylcarbamate.



Chromatography

The derivatized hydroxylamine was separated from other species by reversed-phase partition chromatography with isocratic elution of components using methanol–water (50:50) as mobile phase (Fig. 1). To maximize sensitivity, the dichloromethane solvent was removed by evaporation and the residue reconstituted in a minimum volume of mobile phase prior to HPLC analysis. This volume reduction step results in the appearance of unidentified peaks in the chromatographs, apparently attributable to co-extracted materials and decomposition of methyl isocyanate. HAI was quantitated as HIMU (retention volume, $V_R = 7.8$ ml) by measuring peak height relative to a constant amount ($4.73 \cdot 10^{-5}$ *M*) of N,N-dimethylaniline ($V_R = 14.2$ ml), present as internal standard. A linear relationship was found between HAI concentration and peak height ratio over the range from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*. Linear regression analysis of the data generated the line $y = 0.240$ [HIMU] $- 0.976$ with a correlation coefficient of 0.998. Analysis was carried out with reproducibility of $\pm 5\%$. Accuracy of the determinations, based on the amount of HAI determined by HPLC after extrac-

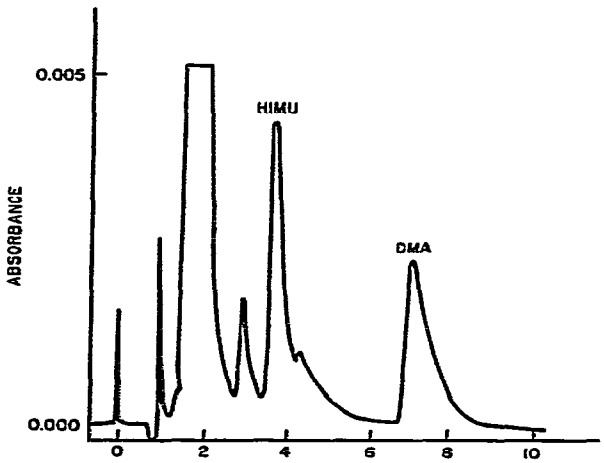


Fig. 1. Chromatography of 1-hydroxy-1-(5′-indanyl)-3-methylurea (HIMU) obtained by extraction of $8 \cdot 10^{-6}$ *M* 5-hydroxyaminoindan (HAI) from liver homogenate and reaction with methyl isocyanate. N,N-Dimethylaniline (DMA; $4.73 \cdot 10^{-5}$ *M*) is present as the internal standard. Components were separated by reversed-phase partition chromatography using methanol–water (50:50) as mobile phase.

tion from liver homogenates and derivatization with methyl isocyanate, was $\pm 4\%$. The minimum concentration of HAI which could be quantitated with this precision and accuracy was $1 \cdot 10^{-7}$ *M* when the analyte was originally dissolved in aqueous buffer solution and $2 \cdot 10^{-6}$ *M* for HAI present in liver homogenates. These limits are all based on 10-μl injection volumes. Larger injection volumes could not be used to enhance sensitivity, because they resulted in an application of greater amounts of interfering substances onto the column, which masked the HIMU peak and decreased column life. Total chromatographic analysis time was less than 9 min.

Methyl isocyanate is a very non-specific reagent and therefore acylatable co-extracted materials will react with it. The products formed will not, however, be

hydroxyureas (unless other arylhydroxylamines are present in the analysis mixture). Analysis of liver homogenate mixtures not containing HAI, but carried through the entire analysis scheme, showed no interfering peaks at the retention volume at which HIMU (V_R = 7.8 ml) and dimethylaniline (V_R = 14.2 ml) elute from the column. Similarly, chromatographic analysis of underivatized (V_R = 12.2 ml) and derivatized (V_R = 10.0 ml) 5-aminoindane, 5-nitrosoindane (V_R = 26.1 ml), 5-nitroindane (V_R = 29.3 ml) and 5,5′-azoxyindane (V_R > 250 ml) (possible metabolic products or metabonates of HAI) did not produce interfering peaks in the region of the peaks of interest. Furthermore, 5-aminoindan (which is also derivatized with methyl isocyanate) and 5-nitroindan, which are the metabolic precursors for HAI, are eluted after the hydroxylamine; therefore, hydroxylamine can be determined in the presence of a large excess of the amine or nitro compound. Under the conditions of methyl isocyanate derivatization, 5-aminoindan was quantitatively converted to the corresponding methylurea, *i.e.* reaction mixtures containing the amine and subjected to isocyanate treatment show no peak at V_R = 12.2 ml; only a peak at 10.0 ml corresponding to the methylurea derivative.

DISCUSSION

A method has been described for the analysis of micromolar levels of the arylhydroxylamine HAI, present in liver homogenates. HAI serves as a model compound having reactivity and solubility characteristics similar to polynuclear aromatic derivatives, but without the proven potent carcinogenicity associated with the latter group. The major difficulty in quantitative analysis of arylhydroxylamines is their instability, *i.e.* at neutral pH they have a half life of *ca.* 20 min; at pH $\geqslant$ 10, the $t_{1/2}$ is less than 2 min[11].

The problem of analysis of such unstable compounds has been handled by either an on-line assay or by chemical conversion of the analyte to a more stable entity. On-line assays have used amperometric transducers[9,16,17] that cannot differentiate between hydroxylamines and aminophenols without prior HPLC separation of components[12]. The procedures are, therefore, either non-specific or time-consuming. Alternatively, arylhydroxylamines have been analyzed by conversion to nitroso[3] or azo[18] derivatives. These products are stable, but are indistinguishable from those formed from alternate amine metabolites or via other routes of biotransformation. The methods are, therefore, non-specific. Furthermore, these procedures are time-consuming and often involve carrying out operations at elevated pH —jeopardizing the stability of the analyte and, therefore, the accuracy of the determination. In this report an analytical procedure is described, in which the arylhydroxylamine is converted to a hydroxyurea by reaction with an isocyanate directly in the extraction solvent. The product is stable, both in the dry state and in solution, so that HPLC analysis of samples can be carried out at a leisurely pace once derivatization is complete. Most importantly, the derivative which is monitored by HPLC is specifically derived from the arylhydroxylamine, *i.e.* the analyte is converted to a product which is different than products formed from other amine metabolites or metabonates. The reagent reacts indiscriminantly with nucleophilic species, but the product that is monitored is only formed by reaction with the arylhydroxylamine. Thus, when

coupled with HPLC separation of components, the method is specific for the arylhydroxylamine.

Sample handling was minimized to insure maximum stability of the underivatized analyte. All operations leading to formation of the hydroxyurea —extraction, aspiration of the aqueous layer and reaction with methyl isocyanate— were carried out in one vessel and as rapidly as possible without compromising efficiency. In this way deterioration of the hydroxylamine was minimized.

The sensitivity limit of the method for the determination of HAI in liver homogenates with methyl isocyanate as the derivatizing agent was $2 \cdot 10^{-6}$ *M*. However, by appropriate selection of isocyanate (*i.e.* R in R–N=C=O), derivatives of hydroxylamines can be formed with radiochemical, strong spectrophotometric, fluorometric or electrochemical properties. Thus, the derivatization reaction can be tailored to the detection system that the investigator wishes to employ and modified to give the sensitivity required for a particular study. This method thus provides advantages for the analysis of arylhydroxylamines over the previously reported chromatographic methods[11,12]: (1) the analyte is converted to a stable species; (2) the hydroxyurea is formed only by reaction with an arylhydroxylamine, so that in this respect, derivatization is highly specific; and (3) the sensitivity of the method is greater than that previously described for analysis with spectrophotometric detection.

ACKNOWLEDGEMENT

This work was supported in part by NIH Grant CA-19288 awarded by the National Cancer Institute (PHS, DHEW).

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 M. Kiese, *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmakol.*, 235 (1959) 351.
4 E. Bamberger, *Ann.*, 424 (1925) 233.
5 A. C. Bratton, E. K. Marshall, D. Babbit and A. R. Hendrickson, *J. Biol. Chem.*, 128 (1939) 537.
6 R. E. Gammans, J. T. Stewart and L. A. Sternson, *Anal. Chem.*, 46 (1974) 620.
7 E. Boyland and R. Nery, *Analyst (London)*, 89 (1964) 95.
8 L. A. Sternson and J. T. Stewart, *Anal. Lett.*, 6 (1973) 1055.
9 L. A. Sternson, *Anal. Chem.*, 46 (1974) 2228.
10 H. Gans, C. S. Lieber and R. Rabin, *J. Pharmacol. Exp. Ther.*, 183 (1972) 218.
11 L. A. Sternson and W. J. DeWitte, *J. Chromatogr.*, 137 (1977) 305.
12 L. A. Sternson and W. J. DeWitte, *J. Chromatogr.*, 138 (1977) 229.
13 E. E. Smissman and M. D. Corbett, *J. Org. Chem.*, 37 (1972) 1847.
14 Y. A. Baskakov, P. I. Svirskaya, G. S. Shvinolerman, N. N. Malysheva, N. B. Vsevolozhskaya, P. V. Tibanov and A. F. Vasiley, *Biol. Aktiv. Soedin.*, (1968) 70; *C.A.*, 71 (1968) 112578.
15 L. A. Sternson and R. E. Gammans, *Bioorg. Chem.*, 4 (1975) 58.
16 L. A. Sternson, *Experientia*, 31 (1975) 268.
17 L. A. Sternson and J. Hes, *Anal. Biochem.*, 67 (1975) 74.
18 Y. Ogata and J. Mibae, *J. Org. Chem.*, 27 (1962) 2048.