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USE OF ION-EXCHANGE CHROMATOGRAPHY IN THE SPECTROPHOTOMETRIC ASSAY FOR THE ANTINEOPLASTIC AGENT, HEXAMETHYLMELAMINE, IN BIOLOGICAL FLUIDS

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SUMMARY

A new, simple, rapid, and precise method for the determination of hexamethylmelamine in human whole blood, plasma, and urine has been described. The method is based upon preliminary column chromatography on anion- and cation-exchange resins followed by ultraviolet absorption spectrophotometry.

INTRODUCTION

Hexamethylmelamine (NSC-13875) (HMM) has demonstrated moderate but reproducible antineoplastic activity in animals[1,2]. On the basis of this activity, HMM was evaluated for human toxicity in clinical Phase I trials[3,4], and a satisfactory dosage schedule for further clinical evaluation in Phase II studies was established[3]. Significant regressions of human neoplasms were recorded[3,5] following therapy with HMM. The mode of action of this drug is unknown[2–5].

To investigate the mechanism of action of HMM and to facilitate studies of the absorption, blood levels, and urinary excretion of this drug by patients, it was necessary to develop a method for the quantitative determination of HMM in biological materials. Although it had been suggested that preliminary purification of the HMM present in blood could be achieved by extraction with organic solvents[3], preliminary studies revealed a low, inconsistent recovery of HMM from blood when the suggested method[3] was employed. It was found that chromatography on anion- and cation-exchange resins could be utilized for the purification of HMM in biological samples prior to quantitative estimation by ultraviolet spectrophotometry. The development of this method for the determination of HMM in blood and urine forms the basis of this report.

EXPERIMENTAL

Materials

HMM (2,4,6-tris(dimethylamino)-*s*-triazine) was provided by the Clinical Branch, Collaborative Research, National Cancer Institute, U.S. Public Health

Service. Dowex 1 (Cl^-), 10 % cross-linkage, 200 to 400 mesh, and Dowex 50W (H^+), 12 % cross-linkage, 200 to 400 mesh, were prepared as described by PRICE[6]. The chromatography columns were made by sealing a glass tube of 1.0 cm outside diameter and 30 cm length to the bottom of a 125 ml Erlenmeyer flask. The columns were operated in groups of 4 to 20 under air pressure distributed with a manifold. A Beckman model DU spectrophotometer (Beckman Instruments Inc., Fullerton, Calif., U.S.A. 92634) with matched quartz cells was used for reading the samples.

Ion-exchange chromatography

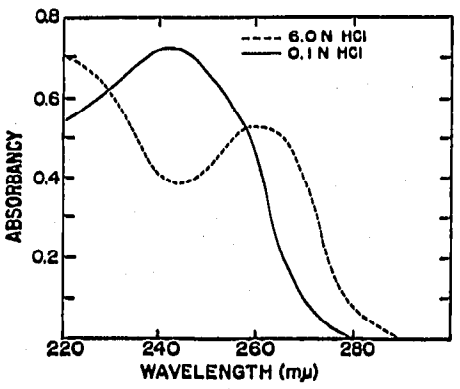
Aqueous solutions, or whole blood, plasma, or urine samples containing known quantities of HMM were acidified with 0.5 ml of 5 *N* HCl and diluted to 25 ml with distilled H_2O. The sample was then applied to a 10-cm resin bed of Dowex 1 (Cl^-) and passed through the column under 1.0 p.s.i. pressure. Then one 25-ml wash of 0.1 *N* HCl was passed through the column and the combined filtrate and wash were pooled. This combined effluent from the Dowex 1 (Cl^-) column was next applied to a 10-cm resin bed of Dowex 50W (H^+) and passed through under a pressure of 0.5 to 1.0 p.s.i. The column of Dowex 50W (H^+) was then washed successively with the following solutions: 50 ml of 0.1 *N* HCl, 100 ml of 0.5 *N* HCl, 100 ml of 1 *N* HCl, 100 ml of 2.4 *N* HCl, 50 ml of 6 *N* HCl, and 100 ml of 6 *N* HCl. Each effluent fraction was collected separately except for the two 6 *N* HCl fractions which were pooled. The pooled 6 *N* HCl effluent was evaporated to dryness *in vacuo*, and distilled H_2O was added and repeatedly evaporated to dryness *in vacuo* several times to remove any remaining HCl. Then 50 ml of 0.1 *N* HCl were added, the residue dissolved, and an aliquot read spectrophotometrically at 242 mμ.

Spectrophotometric determination

Ultraviolet absorption spectra of standard HMM solutions were obtained in 0.1 *N* HCl and 6 *N* HCl (Fig. 1). In the 0.1 *N* HCl solution an absorption maximum was observed at 242 mμ, and in the 6 *N* HCl solution the absorption maximum was at 260 mμ, representing a bathochromic spectral shift for HMM solutions at higher concentrations of HCl. Since the replication of optical density readings was more variable and the peak of maximum absorbance was lower in 6 *N* HCl solutions than in 0.1 *N* HCl solutions, it was elected that all determinations would be made in 0.1 *N* HCl solutions.

Sample preparation

Standard solutions of HMM dissolved in 0.1 *N* HCl were analyzed as described, or were added to whole blood, plasma, or urine samples prior to analysis. Whole blood or plasma samples were prepared by adding standard HMM solutions to 2 ml of whole blood or plasma prior to the addition of 0.5 ml of 5 *N* HCl. Urine samples were prepared by adding standard HMM solutions to 0.25–1.0 % of a 24-h human urine sample, collected in polyethylene bottles containing 20 ml of toluene, prior to the addition of 0.5 ml of 5 *N* HCl. Blanks for whole blood, plasma, or urine were prepared in the above manner except that HMM was omitted from the mixture. These blanks were chromatographed and read against an aqueous solution chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+) as described. All analytical samples were compared with a standard curve of HMM prepared in 0.1 *N* HCl (Fig. 2).



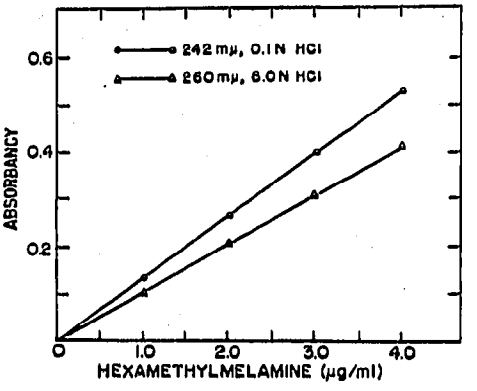
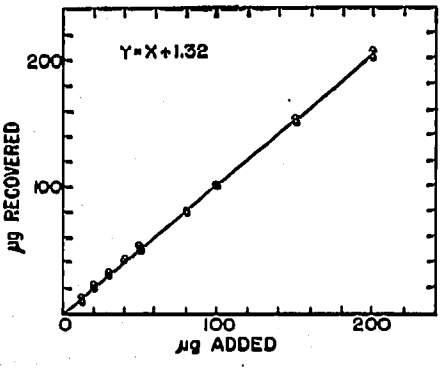


Fig. 1. Ultraviolet absorption spectra of HMM in 0.1 *N* HCl and 6 *N* HCl. A concentration of 5.5 μg/ml of HMM was employed for each solution.

Fig. 2. Standard curves of HMM in 0.1 *N* HCl read at 242 mμ, and in 6 *N* HCl read at 260 mμ. Each curve was calculated by the method of least squares[7] from 50 observations extending over the concentration range of 0.1 μg/ml to 4 μg/ml of HMM. The standard error of the estimate, $s_{y \cdot x}$, for both curves was 0.01 μg/ml.

RESULTS AND DISCUSSION

The ultraviolet absorption spectra of HMM in 0.1 *N* HCl and 6 *N* HCl are presented in Fig. 1. The computed standard curves of HMM in 0.1 *N* HCl and 6 *N* HCl are shown in Fig. 2. The recovery of HMM from Dowex 1 (Cl^-) was quantitative over the range of HMM chromatographed (Fig. 3). When HMM was chromatographed on Dowex 50W (H^+) without prior passage through Dowex 1 (Cl^-), an average of 93.0 % was recovered in the 6 *N* HCl effluent over the range of 10 to 200 μg of HMM. Following chromatography of HMM on Dowex 1 (Cl^-) and Dowex 50W (H^+) the average recovery of HMM was 93.6 % (Fig. 4). Thus, excellent recoveries of HMM



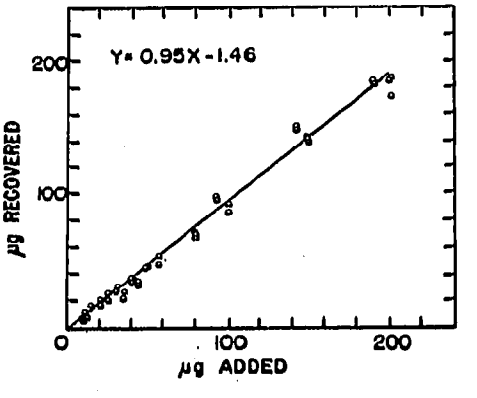


Fig. 3. Regression line for the recovery of HMM in 0.1 *N* HCl after passage through Dowex 1 (Cl^-). The regression line was calculated by the method of least squares[7] from 18 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 2.0 μg. The average recovery of HMM was 102 %.

Fig. 4. Regression line for the recovery of HMM in 0.1 *N* HCl after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 41 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 6.2 μg. The average recovery of HMM was 93.6 %.

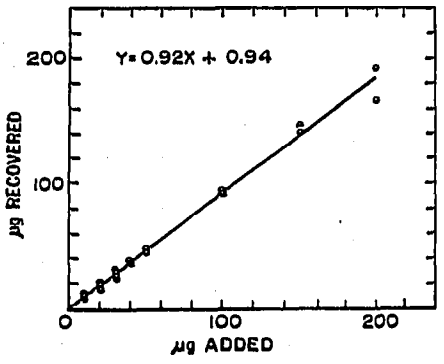
in aqueous solutions could be obtained over a wide range of concentrations with no interference from the blank.

Following the addition of HMM to human plasma samples, excellent recoveries were also observed (Fig. 5). Plasma blank samples containing no added HMM, after being chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+), gave optical density readings of about 0.040 when read at 242 mμ with 0.1 *N* HCl used as the blank. These readings were subtracted from those obtained from samples containing HMM prior to the calculation of recoveries. While the contribution from the plasma blank was small, it appeared that a suitable blank should be analyzed for each sample.

When HMM was added to human whole blood samples and chromatographed as described, average recoveries of 91.7 % of added HMM were obtained over the range of 10 to 200 μg of HMM. The regression line computed for HMM in whole blood was identical to that obtained for HMM in plasma. No preliminary separation of erythrocytes or other formed blood elements was necessary prior to chromatography. Additionally, whole blood blank samples contributed no greater interference than was observed for plasma at 242 mμ.

The recovery of HMM added to human urine samples was comparable to that observed for plasma and whole blood (Fig. 6). However, when urine blank samples containing no added HMM were chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+) resins, optical density readings of 0.160 were obtained with 0.25 % of a 24-h urine sample at 242 mμ using 0.1 *N* HCl as a blank. No suitable way has yet been found to remove these unidentified interfering substances from urine.

The proposed method for the determination of HMM in whole blood, plasma, and urine is rapidly and easily performed. A large number of samples can be analyzed at one time and recoveries of added HMM over a wide range are excellent and reproducible. The contribution of interfering substances to the ultraviolet absorption spectrophotometric determination of HMM in whole blood and plasma is low, but in urine is somewhat greater. However, if appropriate corrections for these interfering



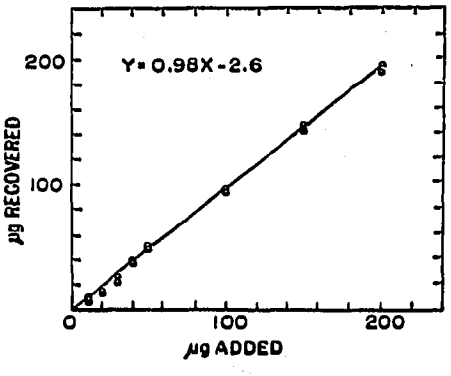


Fig. 5. Regression line for the recovery of HMM added to human plasma after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 22 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 5.1 μg. The average recovery of HMM was 93.4 %.

Fig. 6. Regression line for the recovery of HMM added to human urine after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 20 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 2.3 μg. The average recovery of HMM was 93.7 %.

materials are obtained, excellent, reproducible recoveries can be obtained. This method should facilitate studies of the absorption, blood levels, and urinary excretion of HMM administered to patients.

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