Journal of Chromatography, 145 (1978) 383—392
Biomedical Applications
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 135

RAPID, QUANTITATIVE HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHY OF PSEUDOURIDINE*

KENNETH C. KUO, CHARLES W. GEHRKE and ROY A. McCUNE

Department of Biochemistry, Experiment Station Chemical Laboratories, University of Missouri, Columbia, Mo. 6521 (U.S.A.)

T. PHILLIP WAALKES

Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)

and

ERNEST BOREK

Department of Microbiology, University of Colorado Medical Center, Box 2498, 4200 East Ninth Avenue, Denver, Colo. 80220 (U.S.A.)

(Received October 17th, 1977)

SUMMARY

A rapid, precise, and accurate chromatographic method for the determination of pseudouridine (ψ) in urine by high-performance liquid chromatography (HPLC) has been developed. The ribonucleosides were first isolated with an affinity gel containing immobilized phenylboronic acid. The response for ψ was linear well above and below the range necessary to determine urinary ψ. Good precision was obtained for both matrix-dependent and matrix-independent samples. Supporting experimental data are presented on precision, recovery, chromatographic methods, sample cleanup and application to the analysis of urine samples from normal males and females, and patients with advanced colon cancer. In a comparison of 40 normals with 10 colon cancer patients, 9 of the 10 patients had a ψ:creatinine (Cr) ratio greater than $\bar{x} + 2\sigma$ for the normal population. This HPLC method is now being used extensively in our laboratory as a routine method for determination of ψ in urine from patients with various types of cancer and in chemotherapy response studies. Data are presented on the dynamics of ψ excretion by normal males and females. When the excretion of ψ was normalized with the excretion of creatinine, it was noted that samples collected at random have the same ψ:Cr ratio value as for the 24-h total collection, thus, allowing the use of random samples. The constancy of the ψ:Cr ratio implies that RNA turnover is constant and ψ excretion is independent of diet. Base values are presented for the ψ:Cr

*Contribution from the Missouri Agricultural Experiment Station. Journal series No. 7982.

ratio for normal males 22.6, and females 26.8 (nmoles/μmole). Excellent agreement was found for the values obtained by this HPLC method, with published ion-exchange and gas—liquid chromatography data. This method offers a potential as a screening test for various types of cancer and contributes to biochemical research.

INTRODUCTION

Many RNA researchers are interested in studying the synthesis and regulatory activity of RNA in vivo. Pseudouridine (ψ), the only carbon—carbon ribofuranosyl nucleoside, is found only in RNA and at high concentration. Pseudouridine is not re-incorporated and the lack of catabolic breakdown [1, 2] of ψ should make the excretion rate of this nucleoside an ideal index of RNA activity. Above normal levels of ψ have been found in the urine of patients with various types of cancer [3—6] while recently Borek et al. [7] have reported an elevated tRNA turnover rate in tumor tissue. This suggests that the relationship between RNA metabolism and cancer merits further study.

Several chromatographic methods for the determination of ψ in urine have been developed. Randerath's group developed a thin-layer chromatographic (TLC) method [8] using chemical tritium labeling prior to chromatographic separation and quantitation by liquid scintillation counting of the spots scraped from the TLC plates. This method gives good sensitivity but the sample preparation is laborious and the low resolving power of TLC leads to some problems of incomplete separation.

The high resolution ion-exchange chromatographic (IEC) methods of Uziel [9] and Mrochek [6, 10] require long column regeneration time. Chang et al. [11] have developed a gas—liquid chromatographic (GLC) method for ψ but extensive charcoal cleanup and derivatization for volatility are required. Recently Gehrke and co-workers [12—14] reported on the chromatography and an elegant method for the determination of nucleosides in urine by reversed-phase HPLC with ultraviolet detection at 254 nm.

In this study, we modified our HPLC method for the rapid analysis of only ψ. The resulting chromatographic method is rapid, precise, accurate, and sensitive. The cleanup process was changed to make it more rugged and was also significantly shortened and simplified. The HPLC chromatography requires only 7.5 min per sample. In our laboratory this method is now being used for all routine ψ determinations.

EXPERIMENTAL

Apparatus

All chromatographic studies were conducted with Waters Assoc. (Milford, Mass., U.S.A.) equipment; M-6000A solvent delivery system, model U6K universal injector, and model 440 absorbance detector. The recorder used was a Fisher Recordall Series 5000. The column was a Waters Assoc. μBondapak C_{18}/Porasil (300 × 4 mm I.D.)

The temperature of the column was maintained at 24° using a constant temperature circulating bath, Haake Model FJ (Saddle Brook, N.J., U.S.A.),

connected to an aluminum column jacket. The jacket was composed of two aluminum blocks (24 × 7 × 2.2 cm) precisely grooved to accommodate two columns and a thermometer when bolted together. Each block had two holes (6.0 mm), drilled completely through the block lengthwise, and fitted with Swagelok fittings and copper tubing to allow the constant-temperature water to circulate along four sides of the columns before recycling through the bath. The aluminum column jacket blocks were specially designed and made in the University of Missouri, Science Instrument Shop.

Peak areas, retention times, and concentrations based on an external standard were calculated by a Hewlett-Packard 3352B Laboratory Data System (Hewlett-Packard, Avondale, Pa., U.S.A.). The system consists of a Hewlett-Packard 2100 computer with 16 K memory, 18652A analog to digital converters (A—D), ASR33 teletype, and a 2748B high speed photo reader. The columns used for the boronate gel were glass 5 × 150 mm (Fischer and Porter, Warminster, Pa., U.S.A.) modified by attachment of a 50-ml spherical reservoir to the top of the column. An Eppendorf Model 3200/30 microcentrifuge, Model 3300 rotary shaker, as well as various sizes of Eppendorf pipets (Brinkman, Westbury, N.Y., U.S.A.) were used in the sample cleanup procedure. A Micro Gram-Atic Balance (Mettler, Hightstown, N.Y., U.S.A.) was used to weigh milligram amounts of ψ for the calibration solutions.

Chemicals

The pseudouridine used in these investigations was obtained from Sigma, St. Louis, Mo., U.S.A. Other chemicals were purchased from the following sources. Ammonium acetate and formic acid A.C.S. certified grade (Fisher Scientific, St. Louis, Mo., U.S.A.), ammonium hydroxide, analytical reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.), ammonium dihydrogen phosphate (J.T. Baker, Phillipsburgh, N.J., U.S.A.). Methanol, distilled in glass (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.). All water used for the preparation of buffers and aqueous solutions was purified by a three-step process. The first step was reverse osmosis using an RO-Pure apparatus (DO640 Barnstead Co., Boston, Mass., U.S.A.). A nanopure D1794 four-cartridge water purification system was then used. A charcoal cartridge for adsorption of organics, two mixed bed ion-exchange cartridges for removal of cations and anions, and a filtration cartridge for removal of all particles larger than 0.22 μ were used. Finally, the nanopure water was distilled in a Corning all-glass still (Corning, Corning, N.Y., U.S.A.).

Buffers

A stock buffer concentrate was prepared as 2 l of a 2.0 *M* solution of $NH_4H_2PO_4$. This concentrate was then sterilized by filtering through a Millipore GS-22 filter (0.22 μ) and stored in glass at 4°. A 1-l volume of the working buffer was prepared daily by diluting a 5-ml aliquot of the buffer concentrate with ca. 200 ml of water, adding 10 ml of methanol, diluting the solution to 1 l with water and filtering through a Millipore GS-22 filter. Diluting the buffer concentrate prior to adding the methanol prevents the salt from precipitating out. Stored buffers were maintained in a cold room at 4° and discarded after five days.

Calibration standard solutions

A stock solution of ψ was prepared to give a concentration of about 1.00 mM/ml in distilled nanopure water. The working standard solution was a 10 μM solution. A 60-μl volume of this solution was used to calibrate the chromatography system.

Samples, collection, and storage

The urine samples were collected and maintained at ice temperature. Aliquot samples were frozen and stored at —70°. The normal male control urines were from laboratory personnel with an age span of 20—60 years. The cancer patients selected had advanced colon cancer and at the time of the urine collection the patients were not receiving anti-neoplastic drugs or other anti-tumor therapy. Urine samples from cancer patients were obtained through the courtesy of the National Cancer Institute Solid Tumor Service. Normal female volunteers from the local chapter of the American Cancer Society provided urine samples. Dr. James Hueser supervised the collection. No diet restrictions were imposed.

Phenylboronate affinity gel

An affinity gel with immobilized phenylboronic acid functionality was used for isolation of ribonucleosides prior to HPLC separation and quantitation of ψ [12]. Isolation is the most crucial step in the method. A detailed, stepwise procedure is described as follows:

CLEANUP OF URINE SAMPLES FOR CHROMATOGRAPHY OF ψ

Analytical method

Column preparation

(1) Place ca. 1 ml 0.25 M NH_4 Ac buffer (pH 8.8) in the column (Fischer and Porter No. 274—461, 150 × 5 mm, custom fitted with a 50-ml reservoir).

(2) Slurry the gel in its 0.1 M NaCl storage solution and transfer to the column with a Pasteur pipet (Fisher Scientific, No. 13-678-5B).

(3) Introduce the phenylboronate gel (200—400 mesh) below the surface of the buffer in the column. Care must be taken to prevent the gel from contacting the sides of the reservoir as it adheres to glass.

(4) Allow the column to begin draining and add more gel to a height of 40 mm (bed volume 0.8 ml).

(5) Rinse the gel with ca. 20 ml 0.25 M NH_4 Ac (pH 8.8) buffer. No pressure is used on the column. All solutions are allowed to drain by gravity flow. The flow-rate varies from column to column averaging about 10 ml/h for the 0.25 M NH_4 Ac buffer (pH 8.8) and about 20 ml/h for the 0.1 M HCOOH solution.

(6) Allow the buffer to drain to the top of the affinity gel bed then add 50 ml of 0.1 M HCOOH rinse. The gel expands and contracts depending on the pH and ionic strength of the solution with which it is equilibrated. Formic acid causes the gel to contract visibly but the bed volume is based on the initial volume of the gel in 0.25 M NH_4 Ac buffer (pH 8.8).

(7) Percolate ca. 10 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8) through the column to equilibrate it with this buffer. The gel column is now ready for sample loading when the buffer has drained to the top of the gel bed.

Sample cleanup

(8) The urine sample is thawed and shaken well to ensure sample homogeneity. Draw *exactly* a 0.50-ml aliquot with a 500-μl Eppendorf pipet, and place in a 1.5-ml Eppendorf microcentrifuge tube.

(9) Add 200 μl of 2.5 *M* NH_4 Ac buffer (pH 9.5) to the sample with a 200-μl Eppendorf pipet and mix the sample for 5 min on a vortex mixer (Eppendorf Model 3300 Rotary Shaker).

(10) Centrifuge the sample for 5 min at 12,000 *g* (Eppendorf Model 3200/30 microcentrifuge).

(11) Transfer the sample with a Pasteur pipet on to the column, being careful not to disturb the precipitate.

(12) Add 0.5 ml of the 0.25 *M* NH_4 Ac buffer (pH 8.8) to the sample tube and mix for 5 min on the vortex shaker.

(13) Centrifuge for 5 min at 12,000 *g*.

(14) Transfer the wash on to the column with the same Pasteur pipet.

(15) Follow the sample and wash through the column with 3 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8).

(16) Percolate an additional 3 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8) through the column and after this wash has drained to the top of the gel bed, the column is ready for elution.

Elution of nucleosides

(17) Elute the nucleosides with 5 ml of 0.1 *M* HCOOH. Collect the eluate in a 10 ml volumetric flask.

(18) Bring the sample to volume with glass distilled nanopure water (see reagents section) and mix well by inversion, repeat mixing prior to HPLC analysis.

(19) After elution, strip the columns with ca. 20 ml of 0.1 *M* HCOOH and store in the same solution. Leave ca. 1 ml formic acid above the bed.

(20) To re-use the columns drain off the formic acid and repeat the process from step (7).

Reagents

(1) Ammonium acetate buffer, 0.25 *M* (38.54 g/2 l) with pH adjusted to 8.8 with concentrated ammonium hydroxide. The ammonium acetate used was A.C.S. certified grade from Fisher Scientific and the ammonium hydroxide was analytical reagent grade from Mallinckrodt.

(2) Ammonium acetate buffer, 2.5 *M* (385.4 g/2 l) with pH adjusted to 9.5 with concentrated ammonium hydroxide.

(3) Formic acid, 0.1 *M* (10.33 g concentrated formic acid/2 l). The formic acid used was A.C.S. certified grade from Fisher Scientific.

RESULTS AND DISCUSSION

Reversed-phase HPLC of ψ

A 60.0-μl aliquot of each cleaned sample (equivalent to 3 μl urine) was injected and chromatographed at 1 ml/min with 0.01 *M* $NH_4H_2PO_4$ buffer containing 1% (v/v) methanol on a 4 × 300 mm μBondapak C_{18} column (Waters Assoc.). The 1% methanol buffer gave good separation of the early eluting compounds in urine.

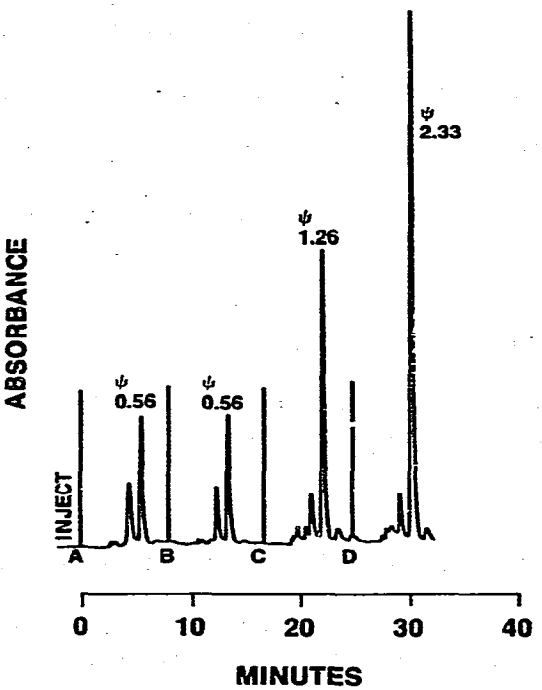

Fig. 1. Reversed-phase HPLC isocratic separation of ψ in urine. Sample: 60 μl of HCOOH eluate, ca. 3 μl urine; (A) 1a, (B) 1b, (C) 2, (D) 2 with 194.82 nmoles ψ /ml added; column: μBondapack C_{18}, 300 × 4 mm I.D.; buffer: 0.01 *M* $NH_4H_2PO_4$, 1% methanol, pH 5.0; flow-rate: 1.0 ml/min; detector: 245 nm, 0.05 a.u.f.s. temperature: 24°; units: nmoles.

Fig. 1 is a series of representative chromatograms. Chromatograms A and B are independently cleaned-up aliquots of urine sample one, which contained 0.56 nmoles of ψ in 3 μl of urine. Chromatogram C is for urine sample two (1.26 nmoles in 3 μl of urine). Chromatogram D represents sample two spiked prior to cleanup with ψ at a level of 194.82 nmoles in 1 ml of urine.

Precision of HPLC determination of ψ

The precision of the HPLC determination of ψ (Table I) was investigated using both standards and samples. Repeated injection of a ψ standard gave a relative standard deviation (R.S.D.) of 0.5% (Table I), and repeated injection of the same urine sample gave a matrix-independent R.S.D. of 1.1%. Then 20 different samples were analyzed with 10 being randomly duplicated. This gave a matrix-dependent R.S.D. of 1.1%. The study demonstrated that

TABLE I

PRECISION OF HPLC CHROMATOGRAPHY OF PSEUDOURIDINE

$n = 10$.

Experiment	$\bar{x}$ (nmole ψ /ml)	σ	R.S.D. (%)
Standard*	96	0.49*	0.51
Pooled control urine** matrix independent	159	1.8**	1.1
Different urines matrix dependent	234	2.6***	1.1

*One standard solution chromatographed ten times.
**Pooled urine passed through gel column then injected 10 times on HPLC column.
***Calculated by pairs using $\sigma = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2P}}$ where x_1 and x_2 are members of the pair, and P = number of pairs = 10.

the HPLC chromatography of ψ is not affected by the sample composition.

The rapidity of the chromatographic determination of ψ is facilitated by the high concentration and narrow peak width of ψ. The other ribonucleosides are at a concentration of at least one order of magnitude lower than ψ, and with the chromatography conditions for ψ (1% methanol) they are eluted as broad bands. This allows samples to be injected every 7.5 min without interference by the compounds still eluting from previous injections.

Linearity of the HPLC determination of ψ

The linear dynamic response range for ψ has been found between 1—50 nmoles. This wide linear range is more than sufficient for routine determination of urinary ψ.

pH Effect on the retention of ψ by the phenylboronate

Of all ribonucleosides ψ is the least strongly retained by the phenylboronic acid gel. A study on the effect of pH on the quantitative retention of ψ by the phenylboronic acid gel was made. Also, we wished to confirm that the gel column used had sufficient capacity to handle 1-ml aliquots of urine.

The pH of a pooled normal urine having a very high creatinine value (16.4 m*M*) was adjusted with 1.0 *M* NaOH and duplicate aliquots were withdrawn at each of the pH's 8.0, 8.5, 9.0, 9.5, 10.0. When 1.0-ml aliquots at pH 8.0 were loaded on the gel columns the values for ψ were 9.0% lower than those at pH 9.0 and greater. Aliquots of 0.50 ml showed no pH effect in the range between pH 8.0 and pH 10.0. The R.S.D. was 5.5% for the 1.0 ml loads and 1.6% for the 0.50 ml load.

The quantitative retention of ψ by the phenylboronic acid gel was dependent not only on the pH of the sample loaded on the column but also on the concentration of compounds in the urine other than the ribonucleosides. These experiments led to the decision to use a 0.5 ml sample and pH adjustment to 9.0 before placement on the 40 × 5 mm boronate column for cleanup.

TABLE II

NORMAL CONTROL VALUES AND DISTRIBUTION FOR PSEUDOURIDINE FROM RANDOM AND TOTAL 24 h COLLECTIONS

$\bar{x}:\bar{x}_{24}$ is a ratio for average value of each (random and total) collection to average value for 24 h total collection.

Time and type of collection	*Male*					*Female*				
	n	$\bar{x}$ (nmoles ψ /μmole Cr)	σ (nmoles ψ /μmole Cr)	RSD (%)	$\bar{x}:\bar{x}_{24}$	n	$\bar{x}$ (nmoles ψ /μmole Cr)	σ (nmoles ψ /μmole Cr)	RSD (%)	$\bar{x}:\bar{x}_{24}$
8 am	10	22.8	2.60	11.4	1.04	15	26.7	4.62	17.3	1.02
10 am	10	22.9	1.65	7.2	1.04	15	28.6	4.40	15.6	1.09
3 pm	10	22.5	2.98	13.2	1.02	15	25.9	3.70	14.4	0.98
24 h	27	22.0	1.68	7.6	1.00	28	26.3	4.9	19.0	1.00
Total*	57	22.4	2.16	9.4	1.02	73	26.7	4.50	16.9	1.02

*The total is the combination of all random and 24-h samples.

Recovery of ψ from gel column

Recovery of ψ for the total chromatography method was determined on a pooled normal urine with a very high creatinine concentration adjusted to pH 9.0 which was confirmed as the optimum pH. Aliquots of 1 ml were spiked with 50 μl of a standard solution containing 95.0 nmoles of ψ. The recoveries were unacceptable with an average of 77.3% on 9 different gel columns with an R.S.D. of 18.2%. The experiment was repeated with 0.50-ml aliquots of urine spiked at the same level. The average recovery increased to 95.4% with an R.S.D. of 5.8%, giving further confirmation of the capacity of the gel column.

The last 3 ml of wash from 1 ml urine loads on the gel column were collected and analyzed. Less than 1% of the ψ was found in this portion of the wash, implying that in the 1 ml loads the ψ was not all retained initially.

Previous capacity studies showed that the gel column quantitatively retained much greater amounts of nucleosides (ca. 30—40 μmoles) than present in 1 ml of urine. Evidently compounds other than ribonucleosides, in the urine exceeded the gel capacity, causing a loss of ψ.

Precision of method for urinary ψ

The precision for a pooled control urine sample taken through the cleanup and chromatographic process was then checked. Nine independent analyses gave an R.S.D. of 2.4%.

Dynamics of excretion

Normal males and females excrete 22.6 and 26.8 nmoles of ψ per μmole of creatinine, respectively (Table II). This reflects a 20% lower excretion of ψ and a 30% lower excretion of creatinine by females. The pseudouridine coefficient (μmoles ψ/kg per 24 h) for males is 4.1 and for females is 3.3; whereas the corresponding creatinine (Cr) coefficient for males is 185 and for females 129 (μmoles Cr/kg per 24 h). The larger RSD, % for the ψ : Cr ratio for female normals might reflect hormonal control of tRNA activity, but further study is required. Our study revealed that the dynamics of excretion of creatinine paralleled those of ψ for a normal population. When ψ was normalized with the excretion of creatinine, the ψ to creatinine ratio values for samples collected at random versus a total 24 h collection were the same (Table II). The constancy of excretion must stem from the constant turnover of RNAs independent of diet. This is of considerable importance in clinical applications where only a random sample can be easily obtained. Also, taking a random sample eliminates the inconvenience and lack of reliability of the 24-h collection. Excellent agreement was found between the normal values obtained by this HPLC method and those of Mrochek et al. [6] by cation-exchange, and Waalkes and co-workers [3, 4] using gas—liquid chromatography.

Excretion of ψ by colon cancer patients

A comparison was made for the ψ:Cr excretion ratio in 40 normals and 10 colon cancer patients and it was found that 9 of the 10 colon cancer patients had a ratio greater than $\bar{x} + 2\sigma$ for the normal population (see Figure 2).

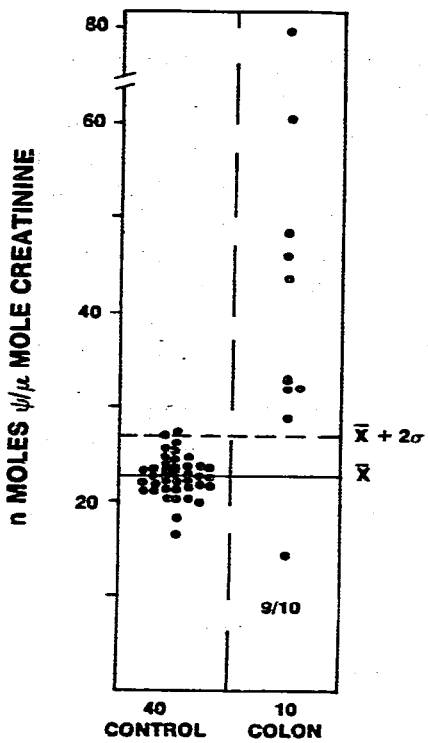

Fig. 2. Elevation of urinary pseudouridine excretion in colon cancer patients.

ACKNOWLEDGEMENTS

The work was supported in part by a contract from the National Cancer Institute, Bethesda, Md. (Contract No. NIH N01 CM 12323).

REFERENCES

1 A. Dlugajczyk and J.J. Eiler, Nature (London), 212 (1966) 611.
2 A. Dlugajczyk and J.J. Eiler, Proc. Soc. Exp. Biol. Med., 123 (1966) 453.
3 T.P. Waalkes, C.W. Gehrke, A.B. Werner, R.W. Zumwalt, C.L.M. Olweny, K.C. Kuo, D.B. Lakings and S.A. Jacobs, Cancer Chemother. Rep., 59 (1975) 721.
4 T.P. Waalkes, C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann and C.G. Moertel, Cancer, 36 (1975) 390.
5 W.S. Adams, F. Davis and M. Nakatani, Amer. J. Med., 28 (1960) 726.
6 J. Mrochek, S. Dinsmore and T.P. Waalkes, J. Nat. Cancer Inst., 53 (1974) 1553.
7 E. Borek, B.S. Baliga, C.W. Gehrke, K.C. Kuo, S. Belman, W. Troll and T.P. Waalkes, Cancer Res., 37 (1977) 398.
8 K. Randerath and E. Randerath, J. Chromatogr., 82 (1973) 59.
9 M. Uziel, C.K. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77.
10 J. Mrochek, W. Butts, W. Rainey, Jr. and C.A. Burtis, Clin. Chem., 17 (1971) 72.
11 S.Y. Chang, D.B. Lakings, R.W. Zumwalt, C.W. Gehrke and T.P. Waalkes, J. Lab. Clin. Med., 83 (1974) 816.
12 G.E. Davis, R.D. Suits, K.C. Kuo, C.W. Gehrke, T.P. Waalkes and E. Borek, Clin. Chem., 23 (1977) 1427.
13 C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, T.P. Waalkes and E. Borek, J. Chromatogr., 150 (1977) 455.
14 C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, T.P. Waalkes and E. Borek, in preparation.