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MONITORING A POTENTIAL CARCINOGEN IN PHARMACEUTICAL FORMULATIONS AT THE LOW PART PER BILLION LEVEL

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF N-NITROSOHEXAMETHYLENEIMINE IN TOLAZAMIDE

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SUMMARY

A method is described for the determination of N-nitrosohexamethyleneimine, a potential carcinogen, in tolazamide bulk drug and pharmaceutical dosage forms. The technique of trace enrichment high-performance liquid chromatography is employed to obtain accurate quantitation of the analyte at levels approaching 1 ppb. Following extraction in diethyl ether and on-line cleanup and enrichment, the nitrosamine is detected by UV at 228 nm.

INTRODUCTION

Tolazamide (TLZ) is a hypoglycemic agent of the sulfonylurea group, which is commonly prescribed for therapy of chronic diabetes. Due to long-term maintenance of therapeutic drug levels, there is a natural concern for the inclusion of any toxic impurities within the dosage form, particularly their cumulative effects. N-Nitrosohexamethyleneimine (NNH) is such an impurity, which is related to other short chain dialkyl nitrosamines of proven or potential carcinogenicity, N,N-dimethylnitrosamine, for example. It is therefore of great importance to monitor the level of this compound in TLZ bulk drug and finished product. Recently, the Food and Drug Administration has established a limit of not more than 100 ppb* of NNH in TLZ bulk drug; the achievement of this low level of NNH has entailed the implementation of further purification procedures during synthesis. The analysis of nitrosamines at the sub-ppm level has been reported by differential pulse polarography[1], gas chromatography (GC)[2] and high-performance liquid chromatography (HPLC) using post-column derivatization[3]. The author is also aware of several unpublished GC methods employing specialized detectors. However, the attainment of low-ppb sensitivity has been difficult by all relevant techniques, and is previously unreported by HPLC with UV detection.

Trace levels of NNH may arise from two sources. The primary genesis of NNH

* Throughout this article the American bilion (10^9) is meant.

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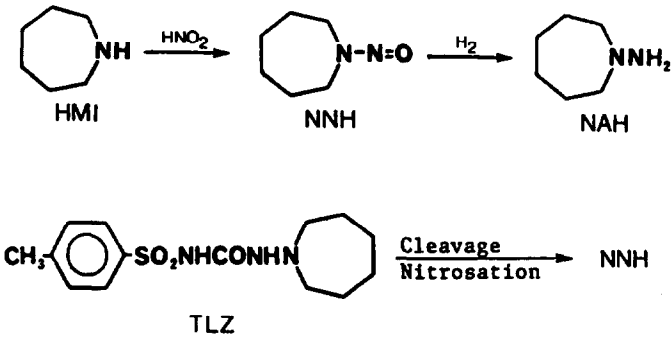

Fig. 1. (Top) synthesis of tolazamide precursor, (bottom) degradation of tolazamide. HMI = hexamethyleneimine, NNH = N-nitrosohexamethyleneimine, NAH = N-aminohexamethyleneimine, and TLZ = tolazamide.

occurs directly during synthesis: NNH is the precursor of N-aminohexamethyleneimine (NAH), which is a reactant in the final synthetic step of TLZ. NNH may also arise through degradation of TLZ (Fig. 1); this route is less likely, since it involves both cleavage of hexamethyleneimine at its attachment to the sulfonylurea group and subsequent nitrosation. The latter reaction may proceed in the presence of nitrites or nitrous anhydride (N_2O_3), which may be present in trace amounts or formed *in situ* by UV irradiation.

The technique of trace enrichment was chosen for the determination of NNH because it offers the benefits of high sensitivity without the need for complex derivatization reactions or specialized detection. Coupled with the appropriate choice of HPLC columns, the technique is capable of providing high specificity of detection in the presence of many potentially interfering compounds. The present procedure allows the cleanup of the sample matrix and the concentration of the desired analyte (NNH) to take place within the sample injection procedure, preceded by relatively simple sample preparation steps. Less than 10 ng of NNH/g of TLZ (= 10 ppb) can be determined reproducibly with a limit of detection (LOD) approaching 1 ppb; the LOD could be reduced even further by an increase in sample weight per determination. The procedure may be performed on a routine basis by a skilled analyst.

EXPERIMENTAL

Reagents and apparatus

Water and methanol were of HPLC grade; diethyl ether, phosphoric acid and sodium phosphate (monobasic and dibasic) were of reagent grade. NNH was used without further purification.

The analytical system consisted of two Hitachi (EM Science, Cherry Hill, NJ, U.S.A.) 655A-11 liquid chromatograph pumps, a Hitachi 655A-71 ternary gradient module, a Hitachi 655-61 gradient controller, a Hitachi 655A variable-wavelength UV detector, and two Rheodyne (Cotati, CA, U.S.A.) 7125 injection valves, equipped for trace enrichment (a 10-port valve may be substituted); the valve plumbing for trace enrichment included a 2-ml injection loop, a 5 cm × 4.6 mm I.D. loop-column packed with 10-μm diameter silica with P-SCX (phenylsulfonate strong cation ex-

changer) bonded phase (ES Industries, Marlton, NJ, U.S.A.); and 3.3 cm × 4.6 mm I.D. analytical column, packed with 3-μm diameter silica with HS-C_{18} bonded phase (Perkin-Elmer, Norwalk, CT, U.S.A.). A pre-column filter, incorporating a 0.5-μm stainless-steel porous frit (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was installed on-line at the analytical column inlet; all critical connections were made with precut stainless-steel tubing of 0.007-in. I.D. The loop-column (for concentration and cleanup of sample), articulating with the 2-ml injection loop at valve A, was eluted by pump A at a flow-rate of 2 ml/min with 20 m*M* sodium phosphate (pH 7.5); the analytical column was eluted by pump B at 3 ml/min with a mobile phase of methanol in 10 m*M* sodium phosphate (pH 4.5). Upon injection at valve B, the eluent from pump B is diverted through the loop-column and backflushes the entrapped sample on the loop-column into the analytical column; the analytical column is eluted isocratically at 10% methanol for 10 min, followed by gradient elution to clear the column of late-eluted material. The column effluent was monitored at 228 nm (0.01 a.u.f.s.; 1.1-s time constant; 5-mm flow-cell path-length).

Mobile phase preparation

Buffer A (2.84 g of dibasic sodium phosphate) was dissolved in about 900 ml of water, adjusted to pH 7.5 ± 0.05 with phosphoric acid, and brought to 1000 ml with water. This buffer was used as the mobile phase of pump A. Buffer B (6.9 g of monobasic sodium phosphate) was dissolved in about 450 ml of water, and brought to 500 ml with water; the pH was adjusted to 4.5 ± 0.05 with phosphoric acid, if necessary.

Buffers and water were filtered through a 0.5-μm Type HA filter (Millipore, Milford, MA, U.S.A.) and degassed under vacuum. Methanol used in mobile phase B was filtered (Type FH, 0.5-μm; Millipore) and degassed under vacuum. For the isocratic portion of the run (10 min), the mobile phase mixed at pump B consisted of buffer B–water–methanol (10:80:10); for gradient elution the mobile phase was changed to buffer B–water–methanol (10:45:45) within 1 min, holding at 45% methanol for 4 min, followed by re-equilibration at initial conditions within about 5 min. (In the absence of gradient-forming devices, a step-gradient may be substituted.)

Sample preparation and procedure

Using a glass mortar and pestle, an amount of TLZ equivalent to 1 g was reduced to a fine powder (*e.g.*, 1 g of TLZ bulk drug; four 250-mg tablets). The sample powder was quantitatively transferred to a 100-ml round-bottom flask with ground-glass joint; 40 ml of diethyl ether was added and the stoppered flask was placed on a mechanical shaker for 30 min. The extract was filtered through Whatman No. 4 filter paper directly into a 250-ml round-bottom flask with ground-glass joint. The extraction flask and residue were washed with two 30-ml portions of diethyl ether, and the flask containing the combined filtrate was placed on a rotary evaporator. The filtrate was evaporated under vacuum at a water-bath temperature of 30°C to a volume of approximately 5 ml.

Using a glass Pasteur pipette, the filtrate was quantitatively transferred to a 25-ml heart-shaped flask with ground-glass joint, rinsing and completing the transfer with two 5-ml portions of diethyl ether. The transfer flask was evaporated just to the point of dryness. The white residue on the walls of the flask was washed down with

exactly 2 ml of water (at this point the sample may be stored in the stoppered flask under refrigeration at 4°C for up to 48 h prior to completion of the analysis). Immediately prior to HPLC analysis, samples were filtered through a low-dead-volume membrane filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) directly into a dry, graduated 2-ml glass syringe, equipped with a blunt needle for HPLC injection. The volume of each sample was recorded, and the sample was injected immediately. The column was eluted as described above. Gradient elution is unnecessary following injection of an NNH standard, which contains no TLZ or other late-eluted components.

RESULTS AND DISCUSSION

The linear range of quantitation was determined at 5–200 ng per injection, corresponding to the equivalent of 5–200 ppb, relative to a 1 g sample of TLZ (correlation coefficient: 0.99970). Depending on attainable signal-to-noise ratio, the minimum detectable level of NNH approaches 1 ng per injection (= 1 ppb) under the reported conditions. The recovery of NNH from spiked placebo over the range of 10–100 ppb was 92.9% with an R.S.D. of 17.0%. Typically, the R.S.D. of replicate samples was *ca.* 10%.

The theoretical plate count (N) for the NNH peak on the analytical column was generally >35000/m, with an N of more than 25000/m for acceptable performance.

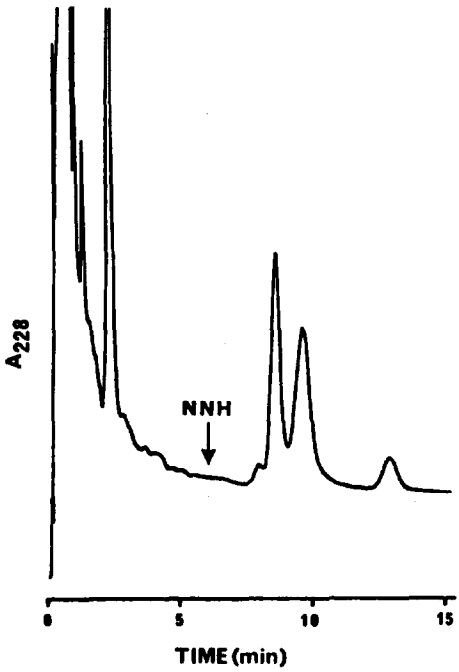

Fig. 2. Chromatogram of tolazamide placebo showing peaks of extractable compounds; arrow indicates retention time of NNH. C_{18} column eluted with 10% methanol in 10 mM sodium phosphate (pH 4.5).

TABLE I

COMPOUNDS RELATED TO NNH THROUGH SYNTHESIS OR DEGRADATION OF TOLAZAMIDE

Compound	*Status**	*RRT***
p-Toluenesulfonic acid	D	0.32
p-Toluenesulfonamide	P,D	0.46
p-Toluenesulfonylurea	P,D	0.48
N-Aminohexamethyleneimine	P,D	*ca.* 5.5
N-*p*-Toluenesulfonyl-N',N'-hexamethyleneurea	S	*ca.* 7
Hexamethyleneimine	P,D	*ca.* 7.5
Tolazamide		*ca.* 11

* D = potential degradation product; P = synthetic precurser; S = side-product.
** Relative retention time; $RRT_{NNH} = 1.00$.

By virtue of its polar nitroso group and its non-polar methylene ring, NNH is soluble in a wide range of solvents, with a relative solvent strength of diethyl ether > water > hexane. Diethyl ether was chosen as the extraction solvent for several reasons: it is the best solvent for NNH, is easy to remove during sample preparation and assures excellent recovery from TLZ.

Chromatographically, NNH exhibits low hydrophobicity and, consequently, weak retention on bonded-phase columns. There are, thus, many potential low-molecular-weight, hydrophilic compounds with similar retention characteristics. In order to achieve high sensitivity, neighboring peaks must be reduced in size by a selective cleanup of extracted matrix components. A strong cation exchange material was chosen for the loop-column for its selective elution properties: NNH is relatively strongly retained (although not by an ion-exchange mechanism) while most other matrix components, including residual TLZ, are weakly retained. As a 2-ml sample is injected into the loop-column and eluted by pH 7.5 phosphate buffer, NNH is retained and concentrated, whereas at least 80% of the other components are washed free of the loop-column. Upon backflush of the loop-column into the analytical column, a purified and enriched NNH fraction proceeds to separation and detection. Fig. 2 shows the peaks of several compounds extracted from a large excess of TLZ placebo tablet which would be present at much greater concentration in the absence of a cleanup procedure; the elution zone of the NNH peak is essentially free of extractable compounds.

A second source of potentially interfering peaks are impurities and degradation products of the active ingredient, TLZ. All available, identified impurities were found not to interfere with the present procedure (Table I).

Two further techniques were employed in order to ensure that the NNH peak of the sample is free of contaminants: peak area ratioing at two separate wavelengths (228 and 254 nm), and fraction collection of sample NNH peaks.

For peak area ratios, a second detector, monitoring at 254 nm, was placed in series with the primary detector (228 nm), and its output was recorded on a separate integrator. Peak areas recorded at these two wavelengths were used to calculate absorbance ratios (R): $R = A_{228}/A_{254}$. The mean ratios (± S.D.) obtained from five injections each of NNH standard (50 ng per injection), and TLZ tablet samples were

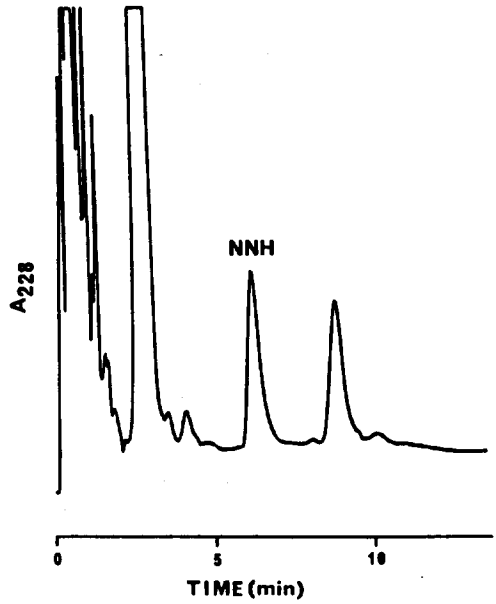

Fig. 3. Chromatogram of NNH in tolazamide tablets. C_{18} column eluted with 10% methanol in 10 m*M* sodium phosphate (pH 4.5); extract of four 250-mg TLZ tablets containing 50 ppb of NNH.

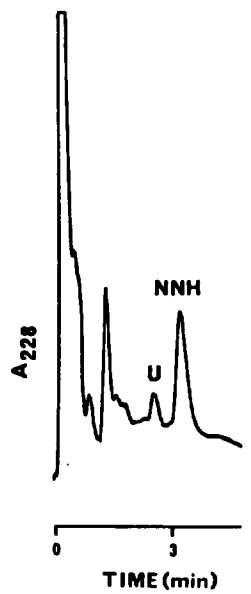

Fig. 4. Chromatogram of NNH fraction from Fig. 3, on Phenyl column eluted with 6% methanol in 10 m*M* sodium phosphate (pH 4.5); U, unidentified degradation product of NNH appearing 24 h after collection.

3.14 ± 0.28 (R.S.D. = 8.98%), and 3.01 ± 0.25 (R.S.D. = 8.35%), respectively. The agreement of these peak area ratios indicates a high degree of peak purity for sample NNH peaks.

The NNH peaks of several TLZ samples were collected at the detector outlet and reanalyzed under conditions of altered column selectivity. In one series of experiments, a Phenyl column (3.0 cm × 4.6 mm I.D., 3-μm particle size; Alltech, Deerfield, IL, U.S.A.) was substituted for the C_{18} column: the mobile phase was prepared with 6% methanol in place of 10% for the C_{18} column; all other parameters were held constant. Fractions collected from samples were injected into the altered system to ascertain the presence of extraneous peaks. It was found that the major peak of all fractions exhibited the same retention time as the NNH standard peak, and >80% peak area was recovered under altered conditions. Interestingly, a second peak appeared in these fractions at relative retention time (RRT) of *ca.* 0.8 (RRT_{NNH} = 1.00) the area of which increased with length of storage between fraction collection and reinjection. This unknown component, which is eluted earlier from a Phenyl column, was shown, after collection and rechromatography, to be eluted later than NNH from a C_{18} column (RRT *ca.* 1.3).

The material in the unknown peak was formed upon storage of NNH in acidic buffer solution and treatment of NNH under oxidizing conditions, indicating that it is a degradation product of NNH. Fig. 4 shows the chromatogram of the NNH fraction collected from the sample illustrated in Fig. 3, and chromatographed the following day on the Phenyl column. The reversal of the elution order and the in-

crease of this product after several days of storage in buffer are noteworthy, since NNH appears to be quite stable in water. The results of the foregoing studies indicate excellent peak purity for NNH quantitated in TLZ bulk drug and finished product.

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