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Fast-LC determination of 1-methyl-1H-tetrazole-5-thiol in human plasma with column-switching sample clean-up

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Abstract: A simple, fast and sensitive method for the quantitative determination of 1-methyl-1H-tetrazole-5-thiol using HPLC is reported. Samples are deproteinated by plasma water filtration and injected into a HPLC system capable of column switching and backflushing the analytical column. The limit of quantitation was determined to be 70 ng ml^{-1} and the limit of detection 22 ng ml^{-1}. Between-day precision (expressed as percent coefficient of variation) of standard curve slopes was ±3.5% with a range of within-day percent coefficient of variations from 0.28 to 1.4%. Recovery and precision of spiked control samples was 96 ± 7% over a concentration range of 630–6300 ng ml^{-1}.

Keywords: *Column-switching clean-up; 1-methyl-1H-tetrazole-5-thiol analysis.*

Introduction

1-Methyl-1H-tetrazole-5-thiol (NMTT) is a degradation product of some cephalosporin or cephamycin antibiotics, including cefazaflur, cefamandole, cefmetazole, moxalactam, cefoperazone, and cefmenoxime. There are currently no data to support a metabolic mechanism for the production of NMTT from these drugs, although Shindo proposed a mechanism of hepatic metabolism with excretion of the NMTT in plasma or bile [1]. However, there seems to be clear evidence linking certain cephalosporins containing the NMTT sidechain with hypoprothrombinemia and platelet dysfunction [2–11]. NMTT is thought to interfere with the biosynthesis of prothrombin precursors [12–18]. The exact mechanism of hypoprothrombinemia associated with certain antibiotics is currently controversial although Lipsky pointed out the similarity of the NMTT dimer structure to disulfiram and the fact that both NMTT and disulfiram inhibited gamma carboxylation of glutamic acid, a necessary reaction in the synthesis of four of the clotting factors [13].

Regardless of the actual mechanism for hypoprothrombinemia and platelet dysfunction, patients with impaired renal function undergoing treatment with antibiotics containing the NMTT sidechain may be at increased risk for this disorder since high concentrations of NMTT would be expected to persist during antibiotic therapy. Because the elimination of NMTT is assumed to be renal excretion and the fact that the degree of protein binding of the NMTT decreases from 36% in normals to 6% in renally impaired subjects [19], monitoring of NMTT levels in renally-impaired subjects administered cefmetazole (a second generation cephamycin antibiotic) was an important element in the safety assessment of this antibiotic. In order to monitor levels of NMTT in clinical plasma specimens of subjects undergoing therapy with cefmetazole, sensitive and specific methodology for its measurement in human plasma needed to be developed. A solid phase extraction procedure [20] and a protein precipitation procedure with trichloroacetic acid in methanol [21] were tried but did not afford adequate sensitivity before being overcome by background interferences. Another procedure using plasma water filtration [19] was limited by analytical sensitivity (250 ng ml^{-1}). A more recent method [22] reports solid phase extraction followed by reversed phase chromatography. While this method is useful for both urine and plasma, it lacks the precision and simplicity of the procedure reported here. This report describes a method using plasma water filtration for sample preparation, improved chromatog-

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raphy conditions, and column-switching sample clean-up, resulting in a more sensitive and precise procedure than has heretofore been published for monitoring systemic concentrations of NMTT.

Materials and Methods

Sample preparation

Calibration standards were prepared in blank normal human plasma (Plasma Alliance, Knoxville, Tennessee) by accurately weighing about 0.7 mg of NMTT (Sankyo Company Limited, Tokyo, Japan) into a 10 ml volumetric flask. Two to three millilitres of plasma were added and the flask was gently swirled until the NMTT was dissolved. The flask was then diluted to the mark with blank plasma to yield a stock standard of 70 μg ml^{-1}. One to one serial dilutions were then made in 25 ml volumetric flasks to provide standards ranging from 35 μg ml^{-1} to 70 ng ml^{-1}.

Control standards were prepared in blank human plasma by accurately weighing about 1.6 mg of NMTT into a 10 ml volumetric flask and dissolving and diluting with human plasma to yield a 160 μg ml^{-1} stock. A working stock of 16 μg ml^{-1} was prepared by pipetting 5 ml of the 160 μg ml^{-1} stock into a 50 ml volumetric flask and diluting with human plasma. Appropriate dilutions of this working stock control standard were made in plasma to yield controls of approximately 6.4 μg ml^{-1}, 1600 ng ml^{-1}, and 640 ng ml^{-1}. Calibration standards and controls were aliquoted into 4 ml glass vials and frozen at −20°C until analysis.

Sample preparation consisted of thawing calibration standards, controls, and subject unknowns and placing approximately 0.5 ml into Amicon Centrifree (Amicon Corp., Danvers, Massachusetts) filters. The filters were then centrifuged at 3000 r.p.m. (1255g) for about 90 min in a fixed-angle rotor at 4°C (Sorvall RT6000, DuPont Instr., Kennett Square, Pennsylvania). Forty microlitres of the filtrate (plasma water) were then hand injected into the HPLC system.

Apparatus

The HPLC consisted of a Hypersil C_{18} 3 μm column (Shandon Southern Instr., Sewickley, Pennsylvania), 4.6 × 30 mm with a mobile phase of buffer–acetonitrile (95:5, % v/v) (Burdick & Jackson, Muskegon, Michigan) pumped at 1 ml min^{-1}. The buffer consisted of 5 mM tetrabutylammonium phosphate (Eastman Chemicals, Rochester, New York) and 0.058 M NaH_2PO_4 (pH 5.75). The column eluant was monitored at 240 nm using a Kratos Model 773 variable wavelength detector (ABI Analytical, Kratos Division, Ramsey, New Jersey) for 10 min with peak heights measured for quantitation. Following elution of the NMTT, a pneumatically driven high pressure switching valve (Model CV-6-HPax, Valco Instruments Co., Houston, Texas) reversed the flow on the column. The column was then back-flushed at 2 ml min^{-1} for 8 min with a rinse containing buffer (as above)–acetonitrile (75:25, %v/v). The valve then returned to the starting position and the column was re-equilibrated with mobile phase for 3 min. Under these chromatographic conditions NMTT eluted at approximately 6–7 min. Total cycle time was 21 min.

Results

Optimization of mobile phase

Previous methodologies [19, 21] utilized mobile phases with pH values of 6.5–7.75 with organic solvent compositions of about 15–20%. Reduction of the organic solvent proportion (acetonitrile) to 5% resulted in longer column retention of NMTT and better separation of NMTT from endogenous components. After examining the separation of NMTT from endogenous components with mobile phases of pH 5, 5.5, 5.75, 6, 6.5 and 7 we determined that optimum chromatography was achieved with a pH of 5.75; this mobile phase moved one background peak further from the NMTT than the other pH values tested, and in addition, this pH was close to the pH optimum (5.5) for cefmetazole stability.

On-line sample clean-up utilizing column switching

Because each specimen containing NMTT would most likely contain cefmetazole (at much higher levels), it was important to provide a means of removing cefmetazole from each specimen. Use of short columns and high flow rates (so called "Fast LC") reduced chromatography time by more than half when compared against chromatography with a 25 cm, 5 μm column. Sufficient theoretical plates were present with the 3 cm column to achieve excellent separation of NMTT and

endogenous components present. However, using the 3 cm column without the column-switching procedure, cefmetazole had a retention time of approximately 63 min. Thus, on-line column switching proved to be a reliable and efficient approach to effectively remove cefmetazole from the sample. Figure 1 shows typical chromatograms of plasma sampled 5 h following three 2 g doses of either placebo (saline), cefmetazole, or cefoperazone. NMTT levels were 0, 300 and 1484 ng ml^{-1} for the respective treatments. The clean background of the placebo subject specimen in the area of NMTT retention illustrates the power of the clean-up procedure. Additionally, since these subjects had been dosed with 6 g of parent drug, it can be seen that there are no apparent background changes, further illustrating complete removal of the parent drug from the chromatographic system.

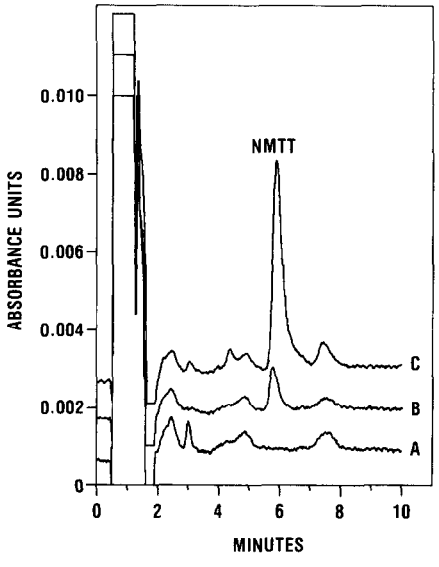

Figure 1
Chromatogram of plasma from a placebo subject (A), a cefmetazole subject (B), and a cefoperazone subject (C), 5 h after the third dose. Subjects were dosed three times *i.v.* with 2 g of either saline, cefmetazole, or cefoperozone, over a 14 h period.

Limit of detection and quantitation

The limit of detection (LOD) for NMTT was estimated by determining the concentration equivalent to approximately three times the detector noise of the low standard (r.m.s. noise) for each analysis and averaging the results. With this method an LOD of 7 ± 2 ng ml^{-1} (0.28 ng on column) was calculated.

The limit of quantitation (LOQ) was established by calculating the level at which the interday coefficient of variation of the calibration curve standards began to exceed 25%. For this method a level of 70 ng ml^{-1} (2.8 ng on column) fits this criterion.

Calibration curve precision

Intercepts for the best-fit linear regression analysis were not significantly different from zero ($p > 0.05$), therefore, linear regression through-the-origin slopes were used for all calculations. Interday coefficients of variation for peak heights of calibration curve standards are given in Table 1. The through-the-origin slope had a between-day RSD of 3% while within-day RSDs on the origin slope ranged from 0.22 to 1.4%. The average correlation coefficient for 11 standard curves was 0.9996 with a range of 0.9981–1.000.

Control accuracy and precision

Controls for NMTT were prepared as

Table 1
Standard calibration curve precision

	Peak height			
Theor. conc. (μg ml^{-1})	*N*	Mean	S.D.	C.V. (%)
0.07057	11	0.33	0.08	26
0.1411	11	0.61	0.13	21
0.2823	11	1.30	0.17	13
0.5645	10	2.7	0.3	13
1.129	11	5.8	0.3	6
2.258	11	12.1	0.8	7
4.516	11	25.0	1.3	5
9.032	11	49.0	2.2	5
18.06	11	102	4	4
36.13	8	206	10	5
72.26	7	402	14	3
Origin slope	11	5.58	0.19	3

Table 2
Control recovery and precision

Concentration ($\mu g\ ml^{-1}$) Label	Found	*N*	Percentage of label (%)	C.V. (%)
0.6307	0.58	19	92	7
1.577	1.51	17	96	6
6.307	6.3	18	99	5

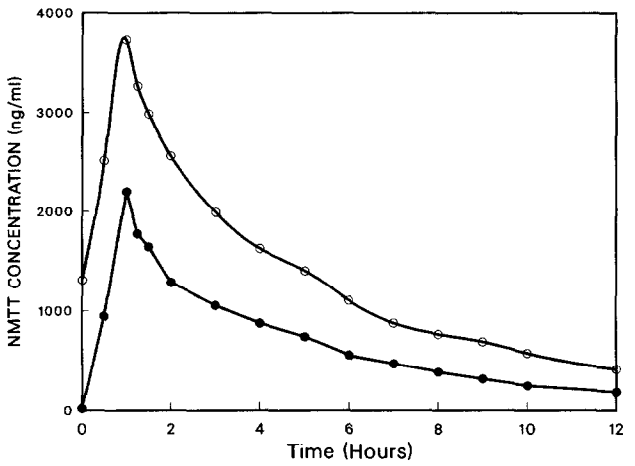

Figure 2
Concentration–time profiles of plasma from a subject dosed with 1 g cefmetazole, given 20 times over six days. The solid circles represent the day 1 first dose blood levels and the open circles represent the day 6 (twentieth dose) blood levels.

described in the Materials and Methods section. Control accuracy and precision was determined by preparing and assaying duplicate controls with each set of unknowns and calibration standards. Excellent recoveries were obtained for controls as shown in Table 2. Overall recovery was 96 ± 7% for all controls combined.

Utility of the method

The method represented by the chromatograms shown in Fig. 1 has also been used on specimens from subjects dosed with moxalactam, another NMTT containing antibiotic, with similar, clean chromatography being obtained. The power of the column-switching clean-up has thus been proven for three different NMTT containing antibiotics. Figure 2 illustrates the concentration–time profile of NMTT in a normal subject dosed with 1 g of cefmetazole, 20 times over six days. As expected, NMTT concentrations in the plasma increased almost two-fold between day 1 and day 6.

These data establish this method as a sensitive, simple, fast, and accurate procedure for determining NMTT levels in human plasma following doses of antibiotics containing the 1-methyl-1H-tetrazole-5-thiol sidechain.

References

[1] H. Shindo, *Sankyo Kenkyusho Nempo (Japan)* **31**, 42–48 (1979).
[2] C.R. Smith and J.J. Lipsky, *J. Antimicrob. Chemother.* **11**, 496–499 (1983).
[3] J. Reddy and R.R. Bailey, *NZ Med. J.* **92**, 378–379 (1980).
[4] R.R. Bailey, *Lancet* **i**, 822 (1983).
[5] R.L. Pakter, T.R. Russell, C.H. Mielke and D. West, *JAMA* **248**, 1100 (1982).
[6] J.A. D'Elia, A. Kaldany, D.G. Miller, D.C. Yoburn and W.A. Kaye, *JAMA* **249**, 1565 (1983).
[7] A.P. Panwalker and J. Rosenfeld, *J. Infect. Dis.* **147**, 171–172 (1983).
[8] H.R. Black, M.K. Buening and R.L. Wolen *Lancet* **ii**, 1090 (1983).
[9] M.R. Weitekamp and R.C. Aber, *JAMA* **249**, 69–71 (1983).
[10] H.C. Neu, *Ann. Intern. Med.* **98**, 415–416 (1983).
[11] N.U. Bang, S.S. Tessler, R.O. Heidenreich, C.A. Marks and L.E. Mattler, *Rev. Infect. Dis.* **4**(Suppl.), S546–554 (1982).
[12] J.J. Lipsky, *Lancet* **ii**, 192–193 (1983).
[13] J.J. Lipsky, *PNAS* **81**, 2893–2897 (1984).
[14] J.J. Lipsky, J.C. Lewis and W.J. Novick, *Antimicrob. Agents Chemother.* **25**, 380–381 (1984).
[15] K. Uchida, T. Ishigami and T. Komeno, *Jap. J. Pharmacol.* **35**, 330–333 (1984).

[16] G.F. Smith and J.L. Sundboom, *Thromb. Res.* **33**, 633–644 (1984).
[17] M. Shearer, K. Andrassy, H. Bechtold, P. McCarthy, J. Koderisch and H. Koderisch, *Thromb. Haemost.* **58**(N1), 80 (1987).
[18] N.U. Bang and R.B. Kammer, *Rev. Infect. Dis.* **5**(Suppl. 3), S380–393 (1983).
[19] G.R. Aronoff, L. Wolen, B.D. Obermeyer and H.R. Black, *J. Infect. Dis.* **153**, 1069–1074 (1986).
[20] K. Nakamura, A. Nakagawa, M. Tanaka, H. Masuda, Y. Hayashi and K. Saionji, *Folia Pharmacol. Jap.* **83**, 183 (1984).
[21] J.R. Barbero, E.L. Marino, M. Barrueco and A. Dominguez-Gil, *Rev. Farmacol. Clin. Exp.* **1**(2), 121–124 (1984).
[22] J.J. Schentag, L.S. Welage, J.S. Williams, J.H. Wilton, M.H. Adelman, D. Rigan and T.H. Grasela, *Am. J. Surg.* **155**(5A), 40–44 (1988).
[23] L.H. Keith, W. Crummett, J. Deegan Jr, R.A. Libby, J.K. Taylor and G. Wentler, *Anal. Chem.* **55**, 2210–2218 (1983).

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