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Isocratic high-performance liquid chromatographic determination of thiacetazone by direct injection of plasma into an internal surface reversed-phase column

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Abstract

This report describes the determination of thiacetazone in human and rat plasma by direct-injection high-performance liquid chromatography (HPLC). Plasma filtrate (50 μl) was injected directly into the internal surface reversed-phase (ISRP) mixed-functional phenyl column (Capcell Pak, 50×4.6 mm, 5 μm) and eluted with an aqueous mobile phase containing 7.5% acetonitrile at a flow-rate of 1 ml/min. With UV detection at 322 nm, thiacetazone eluted at 11.0 min whereas endogenous interferences eluted before 5 min. The lower detection limit for a 50-μl sample at a signal-to-noise ratio of 5 was 63 ng/ml, which was several hundred fold lower than its cytotoxic concentrations determined from in vitro cell line studies. At a concentration range of 0.17 to 2.7 μg/ml, the recovery of thiacetazone was 98.0±4.4% (mean±S.D.). The intra- and inter-day coefficients of variation were 3.0±1.4% and 4.2±2.1%, respectively. This method was successfully applied to study the pharmacokinetics of thiacetazone in rats. The direct injection method is simple, requires small sample volume and does not require sample extraction, internal standard, or gradient elution.

Keywords: Thiacetazone

1. Introduction

Thiacetazone (NSC 3550-P, MW 236), a synthetic thiosemicarbazone, inactivates ribonucleotide reductase [1] and is used extensively in the treatment of tuberculosis in developing countries [2–4]. A recent study showed that thiacetazone is active against primary cultures of human prostate cancer cells [5]. A study in our laboratories confirmed the activity of thiacetazone in human prostate cancer PC3 and DU145 cells; thiacetazone produced a sigmoidal concentration-dependent inhibition of cell proliferation with maximal inhibition approaching 100% (unpublished data). Because human prostate cancer is notoriously resistant to chemotherapy, the finding of significant activity of thiacetazone warrants further investigation.

Thiacetazone has activity against *M. Tuberculosis* and leprosy [4,6,7]. Because of toxicity and low potency, it is rarely used in industrialized countries and is not available in the United States [6]. How-

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ever, because of its availability and low cost, this drug is widely used in developing countries at an oral dose of 150 mg daily with isoniazid (300 mg) for 12 to 18 months to treat tuberculosis and leprosy patients [3,6–8].

Despite its discovered antitubercular activity in 1946 and its wide use in developing countries, limited information is available on the pharmacokinetics of thiacetazone in laboratory animals and humans [4,6,9]. The peak plasma concentration and time to reach peak concentration are about 1.6–3.2 μg/ml and 4–5 h in patients, respectively [8]. The apparent plasma terminal half-life is about 13 h [8]. Approximately 20–40% of the drug is excreted unchanged in the urine [6]. No data was available on the bioavailability, plasma clearance, tissue distribution and protein binding of thiacetazone [10].

Simple colorimetric and spectrophotometric methods have been used to analyze thiacetazone. These methods have poor sensitivity (0.3–0.5 μg/ml) and specificity and are incapable of determining the trough (24 h) concentration after oral administration accurately [11–13]. The only reported high-performance liquid chromatography (HPLC) procedure for thiacetazone involves a two-step liquid–liquid extraction procedure, an internal standard synthesized in house and 3 ml of plasma sample [9]. Due to the complexity and large sample volume requirement, the previously described HPLC method is not suitable for large scale routine analysis and small animal pharmacokinetic studies. The purpose of this study was to develop a simple and sensitive analytical method for thiacetazone in small volumes of biological fluids.

2. Experimental

2.1. Chemicals

Thiacetazone was obtained from the National Cancer Institute (Bethesda, MD, USA) and low-binding Durapore centrifugal filtration units (Ultrafree-MC, 0.45 μm pore size) from Millipore (Bedford, MA, USA). All HPLC solvents were of HPLC grade and were filtered and degassed before use.

2.2. Apparatus and chromatographic conditions

The HPLC consisted of an Hitachi Model L-6200A Intelligent pump (Hitachi Instruments, Naperville, IL, USA), a Waters 717 autosampler (Waters Chromatography Division, Milford, MA, USA) with a 200-μl injection loop, an on-line precolumn filter (0.45 μm, Upchurch Scientific, Oak Harbor, WA, USA), an HP 1050 variable-wavelength UV detector set at 322 nm and an HP 1040A diode-array detector (Hewlett-Packard, Avondale, PA, USA) connected in sequence after the variable-wavelength UV detector to monitor the UV spectrum on-line. The HPLC system was controlled by the HPLC Chemstation data system (Hewlett-Packard).

Two different HPLC columns were used in this study. The conventional reversed-phase HPLC column was a NovaPak C_{18} column (150×3.9 mm, 4 μm, Waters Chromatography Division). The direct injection HPLC column was Capcell Pak mixed function phenyl column (MF Ph-1, 50×4.6 mm I.D., 5 μm, Shiseido, Tokyo, Japan), a new type of internal surface reversed-phase (ISRP) column that contains a mixed-functional phase coating on silicone polymer coated silica beads [14].

2.3. Solutions

Thiacetazone (6.8 mg) was dissolved in 25 ml of acetonitrile to give a stock solution concentration of 0.27 mg/ml. The stock solution was diluted in acetonitrile to give secondary stock solutions of appropriate concentrations. Stock solutions (10 μl) were added to 0.99 ml of blank human plasma to give standard plasma samples or to 50% acetonitrile to give reference solutions, with concentrations ranging from 0.17 to 2.7 μg/ml.

2.4. Animal protocol

Two Copenhagen male rats (Harlan Biomedicals, Dawley, OH, USA), weighing 298 and 335 g, were housed in metabolic cages and had access to food and water ad lib. One day before the experiment, rats were anesthetized with ether and a permanent catheter (silastic tubing, 0.020″ I.D.×0.037″ O.D.) was implanted in the right jugular vein for dose adminis-

tration and sample collection. Intravenous dosing solutions of thiacetazone were prepared in 30% propylene glycol in physiologic saline (v/v) at a concentration of 2.28 mg/ml. The dose (2.5 mg/kg) was administered over 0.5 min, between 8 and 10 am. Serial blood samples (100 to 250 μl) were withdrawn through the venous catheter and the plasma fractions were obtained by centrifugation at 13 000 *g* and 22°C for 1 min. Plasma samples were stored at 4°C and protected from light.

2.5. Sample preparation

For direct injection HPLC, plasma samples (250 μl) were filtered using the Ultrafree-MC centrifugal-driven microporous filters with a pore size of 0.45 μm at 1200 *g* for 15 min to remove particulates. About 100–150 μl of plasma was recovered. The filtered plasma samples (50 μl) were injected directly into the ISRP column.

2.6. Assay validation

The absolute recoveries at nine different concentrations were determined by the ratio of the peak height obtained from injections of same volumes of plasma samples and reference solutions with the same concentrations. Intra-day variation was determined from results obtained from five analysis of the same sample on the same day, and the inter-day variation from injecting the same sample on five different days. Assay accuracy was determined at five different concentrations, by comparing the determined concentration with the nominal concentration.

2.7. Data analysis

The chromatographic parameters, i.e. capacity factor (k'), peak symmetry and theoretical plate count, were calculated by standard methods [15]. The plasma concentration–time data of thiacetazone were analyzed by a standard two-compartment open model using the NLIN routine of SAS (Statistical Analysis System, Cary, NC, USA).

3. Results and discussion

3.1. Elution of thiacetazone from conventional reversed-phase column

To determine the retention and elution of thiacetazone on conventional reversed-phase HPLC columns, we first established the elution profile of thiacetazone with varying strengths of mobile phase. The optimal capacity factor, i.e. k', of 5 to 10 can be achieved with an aqueous mobile phase of 10–14% acetonitrile on the NovaPak C_{18} column (Fig. 1). The peak width ranged from 0.1–0.4 min using mobile phase compositions of 10–35% acetonitrile, with a peak symmetry of 0.8 and a theoretical plate count of about 9600. The on-the-fly UV spectrum showed a maximum absorption at 322 nm (Fig. 2).

3.2. Requirements for direct injection HPLC analysis of plasma sample

ISRP columns have been used as a sample clean-up column in multi-dimensional HPLC applications

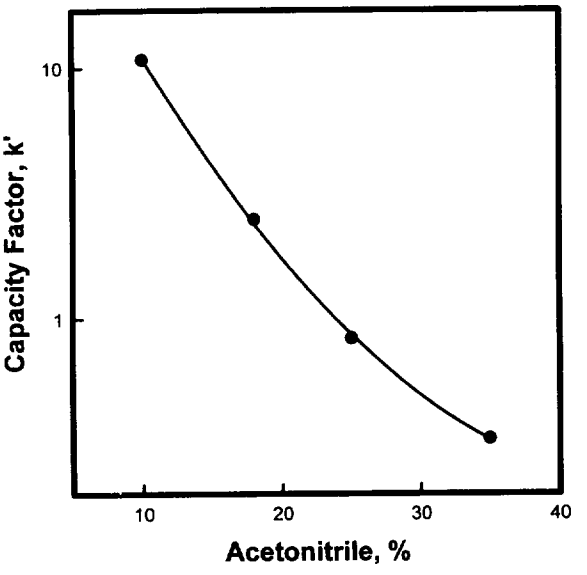

Fig. 1. Elution of thiacetazone on a NovaPak C_{18} column. Capacity factor as a function of mobile phase strength.

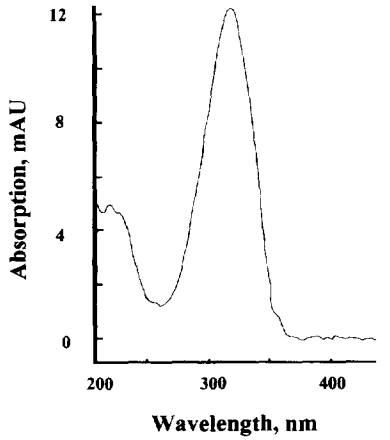

Fig. 2. UV spectrum of thiacetazone. UV spectrum of the thiacetazone peak eluting from Capcell Pak MF Ph-1 column was obtained by on-line diode array detector. The maximum absorption occurred at 322 nm.

[16–20]. However, applications of ISRP column for direct quantitative analysis of biological samples are still limited [21–23]. Successful analysis of plasma samples by direction injection HPLC requires a high specificity in the detection of the analyte in order to diminish interferences and a greater retention of the analyte than the highly bulky and polar plasma matrix components such as proteins. The stationary phase of the ISRP Capcell column consists of an outer surface of hydrophilic polyoxylene groups and an inner surface of hydrophobic phenyl groups. Matrix components such as plasma proteins interact only with the hydrophilic, non-adsorptive outer surface, whereas small drug molecules are selectively retained and are allowed to penetrate and gain full access to the internal hydrophobic phase. Retention of analytes on phenyl groups in the direct injection ISRP column is relatively low compared to C_{18} groups in conventional columns. Hydrophilic compounds with low retention on reversed-phase columns, e.g., 5-fluorouracil, cannot be separated sufficiently from the front peak even without organic solvent (unpublished results). On the other hand, to avoid precipitation of protein in the ISRP columns, the content of organic solvents cannot exceed 20%. We previously reported a direct injection method for mitomycin C in human plasma samples, where mitomycin C was eluted from the ISRP column with 100% water and detected at 365 nm [23].

3.3. Elution of thiacetazone from direct injection ISRP column

The retention of thiacetazone on the ISRP column was lower than on the C_{18} column. Hence, a lower strength mobile phase was used. At a flow-rate of 1 ml/min, thiacetazone eluted at 14.1 min with a mobile phase of water–acetonitrile (95:5, v/v) and 10.9 min with water–acetonitrile (92.5:7.5, v/v). Under these conditions, the eluting thiacetazone peak was broader (1.0 to 1.2 min) than that in the conventional C_{18} column, with a theoretical plate count of 888. The lower plate count was due to the shorter length of the ISRP column (50 mm as compared to 150 mm for the conventional C_{18} column) and the difference in their stationary phase particle size (5 μm for the ISRP column and 4 μm for the conventional C_{18} column). Under these conditions, endogenous interferences in plasma eluted within 5 min as a large peak tailing off gradually. The elution of this front peak showed little dependence on the mobile phase strength ranging from 0 to 15% acetonitrile, which suggests insignificant interaction of compounds in the front peak with the reversed-phase inner surface of the column. However, the peak size and tailing of the front peak diminished significantly when the absorption wavelength was increased above 250 nm, as shown by the on-the-fly UV spectrum (data not shown). Using detection at 322 nm, thiacetazone was baseline separated from the endogenous interferences (Fig. 3). The column was stable after about 200 injections, equivalent to about 10 ml of plasma, with no obvious signs of column deterioration.

3.4. Assay validation

The baseline noise was about 0.025 to 0.035 mAU. At a signal-to-noise ratio of 5, the minimum detection limit from a 50-μl injection of plasma sample was 63 ng/ml. This concentration is about 400-fold lower than the concentration needed to produce 50% cytotoxicity in human prostate PC3 and DU145 cells (unpublished data), and about 50 fold

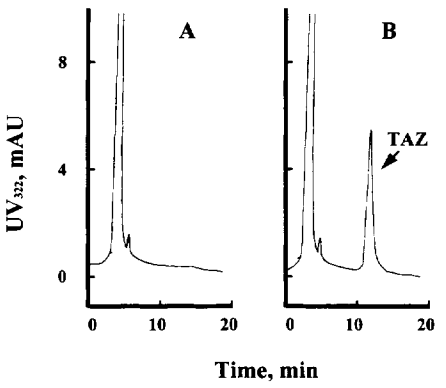

Fig. 3. Elution of thiacetazone on ISRP Capcell Pak MF Ph-1 column. Plasma filtrates were injected into the ISRP column. (A) Blank human plasma (50 μl); (B) human plasma (50 μl) spiked with 2.7 μg/ml thiacetazone. The mobile phase was water–acetonitrile (92.5:7.5, v/v) at a flow-rate of 1 ml/min.

lower than the peak plasma concentration in patients taking 2.5 mg/kg oral dose [8].

The standard curve was linear over the range 0.17–2.8 μg/ml. The linear regression line was: peak height=22 093×concentration−549.5, r^2= 0.9998. The assay was reproducible with average intra-day and inter-day coefficients of variation of below 5%, a high precision of 98.6% and a high and consistent recovery (Table 1). The nearly complete recovery eliminates the need of using an internal standard.

3.5. Application in a preliminary pharmacokinetic study in rats

Fig. 4 shows the plasma concentration–time profiles of thiacetazone in two rats. At an intravenous bolus dose of 2.5 mg/kg, thiacetazone showed a biphasic decline in plasma with a clearance of about 1.2 ml/min/kg and a steady state volume of distribution of about 1155 ml/kg. The average distribution and elimination half-lives were about 5.1 and 724 min, respectively.

4. Conclusions

This report describes a direct injection isocratic HPLC method for the analysis of thiacetazone in human and rat plasma. The assay procedure is simple, does not require sample extraction nor the use of an internal standard, requires only a small sample volume (50 μl) and is suitable for large scale routine analysis and pharmacokinetic studies in small animals where the sample size is limited. This

Table 1
Recovery and intra-day and inter-day variations

Concentration (μg/ml)	Recovery (%)	Intra-day variation C.V. (%)	Inter-day variation C.V. (%)	Accuracy (%)
2.7	99.6	3.2	6.5	102.4
2.4	91.5	3.4	2.3	ND
2.1	98.8	3.4	6.6	ND
1.8	107.9	1.5	2.1	ND
1.6	98.0	1.7	1.8	ND
1.4	95.5	1.5	3.6	100.5
0.68	97.0	3.4	3.2	96.8
0.34	98.1	2.4	4.3	97.4
0.17	95.4	6.1	7.3	99.7
Mean	98.0	3.0	4.2	98.6
S.D.	4.4	1.4	2.1	1.78

Recovery was calculated as the ratio of peak heights of thiacetazone derived from drug-containing plasma filtrates and reference solutions. Intra-day variation was determined from results obtained from five analyses of the same samples on the same day and the inter-day variation from injecting the same sample on five consecutive days. Assay accuracy was determined at five different concentrations.
C.V.=coefficient of variation. ND=not determined.

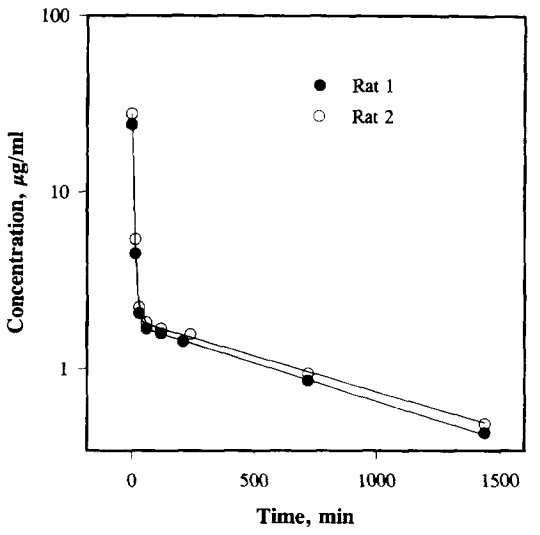

Fig. 4. Plasma concentration–time profiles of thiacetazone in two rats. Thiacetazone (2.5 mg/kg) was administered to two rats by a bolus injection. Lines represent the fitted line using a 2-compartment pharmacokinetic model.

method was successfully applied in a preliminary pharmacokinetic study of thiacetazone in rats.

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References

[1] T. Spector, J.A. Harrington, R.W. Morrison, Jr., C.U. Lambe, D.J. Nelson, D.R. Averett, K. Biron and P.A. Furman, Proc. Natl. Acad. Sci., 86 (1989) 1051.
[2] Y.A. Ipuge, H.L. Rieder and D.A. Enarson, Lancet, 346 (1995) 657.
[3] W. Fox, Chest, 76 (1979) 785.
[4] J. Leowski, Indian J. Chest. Dis. Allied Sci., 24 (1982) 184.
[5] D. Peehl, E. Erickson, L. Malspeis, J. Orr, J. Mayo, R. Camalier, A. Monks, P. Cronise, K. Paull and M.R. Grever, Proc. Annu. Meet. Am. Assoc. Cancer Res., 34 (1993) A2200.
[6] M.H. Cynamon and S.P. Klemens, in L.N. Friedman (Editor), Tuberculosis: Current Concepts and Treatment, CRC Press, Boca Raton, FL, 1994, p. 237.
[7] M. Hooper and M.G. Purohit, Prog. Med. Chem., 20 (1983) 1.
[8] P.J. Jenner, G.A. Ellard and O.B. Swai, Lepr. Res., 55 (1984) 121.
[9] P.J. Jenner, J. Chromatogr., 276 (1983) 463.
[10] M.R. Holdiness, Clin. Pharmacokin., 9 (1984) 511.
[11] J.S. Shohet, Analyst, 99 (1974) 755.
[12] C.J. Shishoo, M.B. Devani and M.G. Shah, Analyst, 98 (1973) 762.
[13] G.A. Ellard, J.M. Dickinson, P.T. Gammon and D.A. Mitchison, Tubercle, 55 (1974) 41.
[14] I.A. el Hag, G. Roos, P.E. Jonsson and U. Stenram, Anticancer Res., 10 (1990) 29.
[15] A. Braithwaite and F.J. Smith, Chromatographic Methods, Chapman and Hall, London, 1985.
[16] J.C. Cherton, C. Loutelier, C. Lange and P. Cassier, Sci. Total Environ., 132 (1993) 381.
[17] A. Puhlmann, T. Dulffer and U. Kobold, J. Chromatogr., 581 (1992) 129.
[18] K.C. Rosenspire, W. Hirth, S. Jurisson, D.P. Nowotnik, W.C. Eckelman and A.D. Nunn, J. Chromatogr., 574 (1992) 119.
[19] S.C. Ruckmick and B.D. Hench, J. Chromatogr., 565 (1991) 277.
[20] Y.Q. Chu and I.W. Wainer, Pharm. Res., 5 (1988) 680.
[21] S.J. Rainbow, C.M. Dawson and T.R. Tickner, J. Chromatogr., 527 (1990) 389.
[22] S.J. Rainbow, C.M. Dawson and T.R. Tickner, Ann. Clin. Biochem., 26 (1989) 527.
[23] D. Song and J.L. Au, J. Chromatogr. B, 676 (1996) 165.