Journal of Chromatography, 526 (1990) 151–157
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5095

Determination of tauromustine and its demethylated metabolites in plasma and urine

JIRI POLACEK*, B. GUSTAFSSON, S. BRANDIN and A. OTTERSGÅRD-BRORSSON

Pharmacia LEO Therapeutics AB, Box 941, S-251 09 Helsingborg (Sweden)

(First received May 3rd, 1989; revised manuscript received November 1st, 1989)

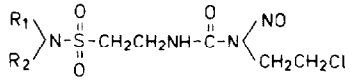
SUMMARY

A sensitive, selective and precise high-performance liquid chromatographic method for simultaneous determination of tauromustine and its demethylated metabolites in plasma and urine has been developed. It is based on solid-phase extraction on C_{18} sorbent and separation on a semipolar column. The analytical procedure is described in detail. The method has been validated with respect to linearity, recovery, selectivity, precision and detection limit. The stability of the determined substances in various media has also been studied.

INTRODUCTION

Tauromustine, 1-(2-chloroethyl)-3-[2-(dimethylaminosulphonyl)ethyl]-1-nitrosourea (TM, TCNU; Fig. 1), is a water-soluble antitumour nitrosourea based on the endogenous aminoethanesulphonic acid taurine. Preclinical studies with TM have revealed its potent antitumour activity against several experimental tumours in vivo and in vitro [1,2], and its efficacy against nitrosourea-resistant tumours [3]. During phase I clinical trials in cancer patients [4,5], who received single oral doses of TM, plasma concentrations of the drug were monitored in 31 patients [6] using a selective and sensitive high-performance liquid chromatographic (HPLC) assay with UV detection, developed in our laboratory [7]. This method was based on liquid–liquid extraction and chromatography on a reversed-phase column. Recent metabolic studies with TM have revealed that demethylation is one of the metabolic pathways in vitro and in vivo [8]. The demethylated metabolites, demethyl tauromustine (DMTM, LS 2724) and didemethyl tauromustine (DDMTM, LS 2715)

0378-4347/90/$03.50



	R_1	R_2
DDMTM	H	H
DMTM	CH_3	H
TM	CH_3	CH_3
IS	CH_3	CH_2CH_3

Fig. 1. Structures of tauromustine (TM), its demethylated metabolites (DMTM and DDMTM) and the internal standard (IS)

(Fig. 1) possess anti-tumour activities similar to that of TM [1]. An HPLC method for simultaneous quantification of TM and its demethylated metabolites in plasma and urine is described in this paper.

EXPERIMENTAL

Materials

Tauromustine, its demethylated metabolites (DMTM and DDMTM) and the internal standard (I.S.) were synthesized at the Pharmacia LEO Therapeutics laboratories [1]. Hexane, methanol and acetonitrile were of HPLC grade (Labscan, Dublin, Ireland), ethyl acetate was of analytical grade (BDH, Poole, U.K.) and 4-bromoacetanilide (BAA) was purchased from Fluka (Buchs, Switzerland). Water was deionized by a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). All other chemicals were purchased from standard sources and used without further purification. Bond-Elut C_{18} extraction columns (100 mg, 1 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Procedure

A frozen plasma sample was acidified with 2 *M* hydrochloric acid, ca. 10 μl/ml, and thawed in a water-bath at room temperature. When just thawed, the sample was thoroughly vortex-mixed and centrifuged at 2200 *g* for 5 min. Without delay, 1.00 ml was transferred to a test-tube containing 0.500 ml of a mixture of I.S. (2.4 μg/ml) and 4-bromoacetanilide (0.4 μg/ml) in 0.005 *M* KH_2PO_4 and 0.50 ml of 0.5 *M* phosphate buffer (pH 6) were added. The mixture was vortex-mixed and applied on a Bond-Elut column, activated by flushing with 2 ml of methanol and 2 ml of 0.5 *M* phosphate buffer (pH 6). The sample was passed through under vacuum, and the sorbent was washed with 1.0 ml of hexane and dried for 3 min under vacuum. The compounds were eluted with two 0.2-ml volumes of hexane–ethyl acetate (40:60, v/v). The eluting solvent was evaporated under a gentle stream of nitrogen at room tem-

perature. The residue was dissolved in 0.1 ml of methanol–acetic acid (999:1, v/v) and 15 μl were injected into the HPLC system. The sample solutions were protected from light during the whole procedure.

Chromatography

The chromatographic system consisted of a Spectra Physics (Santa Clara, CA, U.S.A) SP 8780XR autosampler and an SP 8800 ternary HPLC pump, a Kratos (Westwood, NJ, U.S.A.) Spectroflow Model 757 variable-wavelength UV detector, set at 250 nm, and a Spectra Physics SP 4270 integrator. The HPLC column was an APEX II Cyano (250 mm $\times$ 4.6 mm I.D., 5 μm particle size) matrix cartridge column (Jones Chromatography, Hengoed, U.K.) preceded by an APEX II Cyano (10 mm $\times$ 4.6 mm I.D., 5 μm particle size) precolumn matrix cartridge. The mobile phase was prepared by mixing 60 ml of methanol with 80 ml of acetonitrile and diluting to 1000 ml with 0.005 *M* KH_2PO_4. The pH of the mobile phase was adjusted by addition of 1 *M* H_3PO_4 or 1 *M* NaOH to a chosen value between 4.0 and 6.4. The flow-rate was 1.6 ml/min. All experiments were carried out at ambient temperature.

Calibration

Calibration solutions were prepared using blank plasma or urine. The solutions were processed as described above. Calibration graphs were established by plotting the peak-height ratios of TM, DMTM and DDMTM to I.S. versus the amounts of the substances added in the calibration solutions. The graphs were evaluated by the least-squares linear regression method, and the slope coefficients (k_i) from the regression equations $y = k_i x + b$ were used to calculate the concentrations of TM, DMTM and DDMTM in unknown plasma or urine samples. The second internal standard, BAA, was used for calculation only if evaluation using I.S. was impracticable.

RESULTS AND DISCUSSION

Extraction procedure

Like other nitrosoureas, TM shows optimal stability in slightly acidic solution. Frozen plasma was therefore acidified before thawing, although this was normally not necessary when urine samples were analysed. However, it turned out that interference originating from endogenous sources was minimized at pH ca. 6, therefore the extraction on Bond-Elut sorbent was carried out at that pH. The extraction efficiency was determined using human plasma (P) and aqueous buffer samples (W), spiked with ca. 800 ng/ml TM, DMTM and DDMTM. The samples were treated accordingly and the results were compared with those obtained for aqueous samples directly injected to the HPLC system. The average absolute recovery was $92.6 \pm 2.2\%$ ($n = 27$) for P samples

and 93.2 ± 1.9% ($n = 27$) for W samples, which demonstrates that the recoveries were not influenced by the plasma matrix.

Selectivity

TM and its demethylated metabolites are well separated. Chromatograms obtained for plasma from a cancer patient before treatment and 90 min after oral administration of TM and for human urine spiked with TM and the metabolites are shown in Fig. 2. The method was tested for the presence of interfering peaks originating from various sources. Drug-free plasma from humans, dogs and rats, as well as human urine, were tested for interference from endogenous components. Unidentified peaks interfering with the DDMTM peak appeared on some chromatograms. These disturbances could often be eliminated by adjusting the pH of the mobile phase. Normally, a pH value of ca. 4 was chosen for plasma samples and of ca. 6 for urine samples. The k' values and retention times of the determined substances and internal standards were not influenced by these pH variations. Denitrosated products, which may arise by metabolism or by chemical degradation, as well as other possible degradation products, were not detected owing to lack of UV absorption at the wavelength of measurement. No interference was obtained from metoclopramide, an anti-emetic drug, or from 5-fluorouracil, a drug often used in combination therapy with nitrosoureas.

Linearity

Calibration graphs using seven different concentrations in the range 0–5 μg/ml TM, DMTM and DDMTM in human plasma or urine were established. Nine calibration graphs were obtained over a period of a month, seven of them

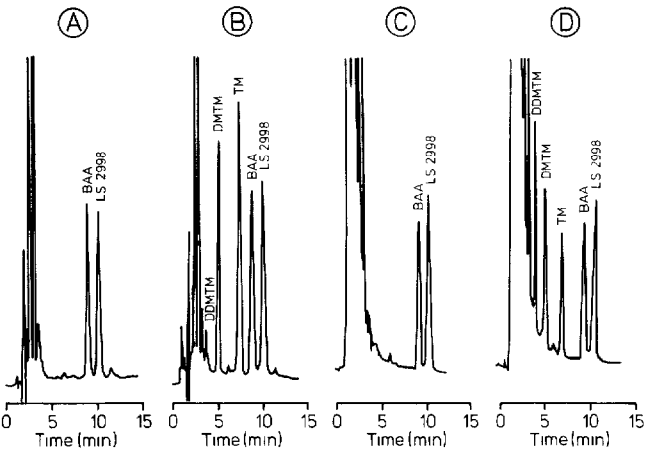


Fig. 2. Chromatograms of plasma from a cancer patient before treatment (A) and 90 min after oral administration of TM (B) and from drug-free human urine (C) and the urine spiked with 400 ng/ml DDMTM, DMTM and TM (D).

from plasma and two from urine. The calibration data were subjected to the least-squares linear regression analysis. Typical regression lines were described by the equations $y_{TM}=1.275x+0.001$, $y_{DMTM}=1.778x+0.004$ and $y_{DDMTM}=2.359x+0.010$, all with $r>0.9999$ (y=concentration in μg/ml).

Precision

Human plasma and urine were spiked with a mixture of TM, DMTM and DDMTM at two different concentrations, ca. 50 and 5000 ng/ml. Six to seven replicates of each were analysed on the same occasion. The coefficients of variation (C.V.) ranged from 1.4 to 5.1% (low concentration) and from 0.5 to 2.1% (high concentration). In another experiment, human plasma and urine were spiked with 480 ng/ml TM, DMTM and DDMTM. Aliquots of 1.0 ml were frozen and stored at ca. −65°C. Plasma samples were analysed in triplicate on six different occasions within a period of eight days. Urine samples were analysed on two different occasions ten days apart. The results are presented in Table I.

Detection limit

Initially, the detection limit was estimated roughly from the signal-to-noise ratio in chromatograms obtained in the normal way. In order to get more exact values, aqueous buffered solutions containing TM, DMTM and DDMTM in concentrations near the estimated detection limit were prepared, frozen and stored at ca. −65°C. Four to five samples were then analysed on four occasions together with plasma samples, routinely analysed in our laboratory. The detection limit, expressed as three times the standard deviation, was ca. 2 ng/ml for TM and ca. 5 ng/ml for DMTM and DDMTM.

TABLE I

INTER-DAY PRECISION OF THE HPLC METHOD FOR TM AND ITS METABOLITES

The S.D. and C.V. were calculated from triplicate analyses.

Compound	Matrix	Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	n
TM	Plasma	480	479 ± 7	1.5	6
	Urine	492	481 ± 4	0.9	2
DMTM	Plasma	481	484 ± 10	2.0	6
	Urine	491	481 ± 3	0.6	2
DDMTM	Plasma	479	478 ± 19	4.0	6
	Urine	493	481 ± 4	0.8	2

TABLE II

STABILITY OF TAUROMUSTINE AND ITS ANALOGUES IN SOLUTION

Results from the linear regression analysis, $\log y = kx + \log b$. T_2 (T_{max}, T_{min}) = time after which the content of the drug has decreased about 2% (95% confidence limits).

Matrix	Temperature (°C)	k (h^{-1})	$\log b$	r	S.D	T_2 (h)	T_{max} (h)	T_{min} (h)	n
0.005 *M* KH_2PO_4 (pH 5.2)	5	−0.00054	2.0024	−0.97087	0.002601	16.2	19.2	14.0	13
0.5 *M* Phosphate buffer (pH 6)	6	−0 001626	1.98415	−0.93726	0.010281	5.4	6 6	4 6	21
0.1% Acetic acid in methanol	25	−0.000415	1.99924	−0.7650	0.002723	21.1	25.2	18.2	44

Stability

Nitrosoureas are labile compounds that are easily degraded when in solution. The rate of the degradation is pH-dependent [9]. Therefore the stability of TM and its analogues in various media used during the analytical procedure was studied. Solutions were kept at constant temperature, protected from light, and analysed after different times of storage, using HPLC or UV spectroscopy. The same rate of degradation of TM, DMTM, DDMTM and I.S. was presupposed, and all the experimental data were pooled. Semi-logarithmic plots of the percentage of intact substance as a function of time were established, and the data were evaluated using the least-squares linear regression analysis (line equation $\log y = kx + \log b$). The rate of degradation of TM and its analogues is demonstrated by the calculated slopes of degradation curves, k, and by the time (T_2) after which the content has decreased by ca. 2% (Table II).

The stability of TM and its metabolites in plasma at low temperature was also studied. Samples containing ca. 500 ng/ml TM, DMTM and DDMTM in plasma from various sources (human, dog and rat) were prepared, frozen and stored at ca. −65 °C. Samples were analysed on the day of preparation, on the following day and then after various times of storage. The results of this experiment indicate that TM and the metabolites in plasma are stable for at least six months when kept at temperatures below −65 °C.

CONCLUSIONS

The described procedure provides good linearity, recovery, selectivity, sensitivity and precision for the determination of tauromustine and its demethylated metabolites in plasma and urine. The method is therefore suitable for pharmacokinetic studies with tauromustine in animals and humans.

ACKNOWLEDGEMENTS

The authors thank Arne Olsson for valuable comments and M.-B. Hansson for help with the preparation of the manuscript.

REFERENCES

1 B. Hartley-Asp, P.I. Christensson, K. Gunnarsson, P.O. Gunnarsson, G. Jensen, J. Polacek and A. Stamvik, Invest. New Drugs, 6 (1988) 19.
2 H. Roed, L.L. Vindelöv, M Sprang-Thomsen, I.J. Christensen and H.H Hansen, Cancer Chemother. Pharmacol., 19 (1987) 315.
3 M.C. Bibby, J.A. Double and C.M. Morris, Eur. J. Cancer Clin. Oncol., 24 (1988) 1361
4 J. Vibe-Petersen, E. Bork, H. Möller and H.H. Hansen, Eur. J. Cancer Clin. Oncol , 23 (1987) 1837.
5 J.F. Smyth, J.S. Macpherson, P.S. Warrington, M.E. Kerr, J.M. Whelan, M.A. Cornbleet and R.C.F. Leonard, Eur. J Cancer Clin. Oncol., 23 (1987) 1845.
6 P.O. Gunnarsson, J. Vibe-Petersen, J.S. Macpherson, P.S. Warrington, J. Polacek, M Ellman, H.H. Hansen and J.F. Smyth, Cancer Chemother. Pharmacol., 23 (1989) 176.
7 J. Polacek, P.O. Gunnarsson and S. Brandin, J. Chromatogr., 425 (1988) 424.
8 J. Seidegård, L. Grönqvist and P.O. Gunnarsson, Biochem. Pharmacol., in press.
9 A.G. Bosanquet, Cancer Chemother. Pharmacol., 14 (1985) 83.