Journal of Chromatography, 419 (1987) 381-387
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3719

Note

Qualitative and quantitative analysis of a new sulphur-containing nitrosourea in blood by high-performance liquid chromatography

P. LABARRE*, D. GODENECHE and J.C. MADELMONT

INSERM U 71, B.P. 184, 63005 Clermont-Ferrand Cedex (France)

K. TRIANA and G. MATHE

Institut de Cancérologie et d'Immunogénétique, Hôpital Paul Brousse, 94804 Villejuif Cedex (France)

and

A. VEYRE

Laboratoire de Biophysique Médicale, Faculté de Médecine, 28, Place Henri Dunant, 63001 Clermont-Ferrand Cedex (France)

(First received December 12th, 1986; revised manuscript received March 24th, 1987)

The nitrosoureas show antitumour activity against a broad spectrum of animal and human neoplastic diseases. To date, the most widely used in cancer chemotherapy are 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea [1-3].

More recently, CNCC, an isomeric mixture of 2-chloroethyl nitrosocarbamoylcystamine, was synthesized. This compound showed very promising antitumour activity in experimental tumour models [4,5]. Metabolic studies in different animal species indicated that CNCC undergoes an extensive first-pass metabolism [6,7]. Four plasma metabolites were identified in animals and in humans. They are derived from the breakdown of the disulphur bridge of CNCC, followed by the methylation and oxidation of the sulphur atom. Two of the metabolites, N'-(2-chloroethyl)-N-[2-(methylsulphinyl)ethyl]-N'-nitrosourea ($CMSOEN_2$) and N'-(2-chloroethyl)-N-[2-(methylsulphonyl)ethyl]-N'-nitrosourea ($CMSO_2EN_2$) (Fig. 1), show an effectiveness greater than that of the parent compound on several murine tumours [8-11]. Moreover, these water-soluble nitrosoureas display moderate hematotoxicity. To date, only $CMSOEN_2$ has been involved in a phase I clinical trial.

Only a few reports have given information about nitrosourea pharmacokinetics

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

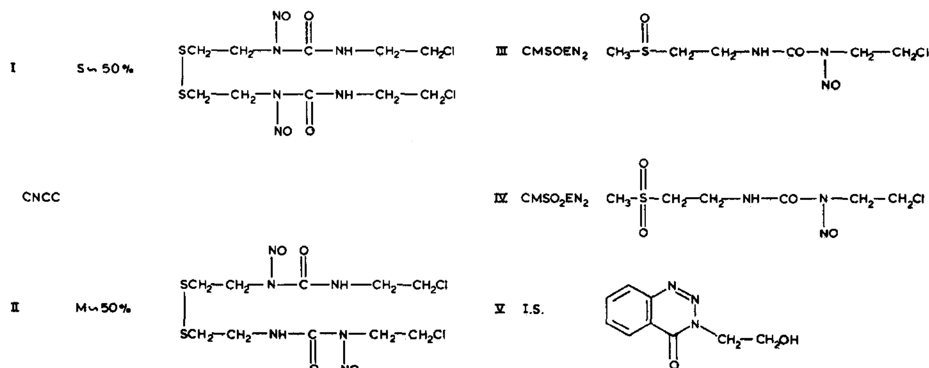


Fig. 1. Structural formulae of isomers of 2-chloroethylnitrosocarbamoylcystamine (I and II), of the two active metabolites $CMSOEN_2$ (III) and $CMSO_2EN_2$ (IV) and of 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3*H*)-one (V).

in humans. Assays included chemical ionization mass spectrometry, high-performance liquid chromatography (HPLC) and gas chromatography [12–14]. Given the instability of this class of compounds in aqueous media [15], rapid, selective and sensitive methods are required for their quantitative determination in biological fluids.

This report describes a simple and selective HPLC technique for the accurate assessment of drug concentrations in whole blood of patients.

EXPERIMENTAL

Reagents

$CMSOEN_2$ was prepared in our laboratory by Madelmont et al. [3,4]. The internal standard 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3*H*)-one (Fig. 1) was kindly supplied by Rhône Poulenc Santé (Paris, France).

All chemicals and solvents were of either analytical or HPLC grade and were used without further purification.

Apparatus

A high-performance liquid chromatograph (Hewlett-Packard HP 1090) with integrated modules was used for all the assays. This consisted of a solvent-delivery system of three metering pumps, an autoinjector, a diode array detector monitoring all wavelengths simultaneously from 190 to 600 nm (DAD HP 1040), an integrator (HP 3392) and a plotter (HP 7470,A). All the modules were controlled by a personal computer (HP 85B) through the HPIB interface. The DAD detector allowed the identification and the evaluation of purity of reference compound peaks by monitoring and plotting three superimposed spectra (upslope, apex and downslope spectra).

Patients sampling

Patients admitted to the department of Professor G. Mathé (Hospital Paul Brousse, Villejuif, France) were given a 15-min intravenous perfusion of

$CMSOEN_2$ (45 mg/m^2). Venous samples (5 ml) were withdrawn at intervals from 5 min to 3 h. Each sample was collected in an heparinized tube, immediately frozen at −80°C and shipped in dry ice by air mail to our institution.

Extraction

To 2.5 ml of blood after thawing were added 0.0625 ml of a solution of internal standard in acetonitrile (2 μg/ml). Then 2 ml of blood spiked with internal standard kept in ice at 4°C were loaded onto a 10-ml plastic syringe filled with 1 g Extrelut® (Merck, F.R.G.). After 10 min, elution was performed with 10 ml of dichloromethane. The extract was evaporated to dryness at 40°C under a stream of dry nitrogen. The residue was dissolved in 0.1 ml of dichloromethane and transferred to a low-volume sample vial.

Recoveries of $CMSOEN_2$ and internal standard were estimated by comparing the peak height of a plasma extract containing a known amount of the two compounds with the peak height of a reference solution.

Quantification

For each sample, the peak-height ratio of the drug to that of internal standard multiplied by the added concentration of internal standard (ng/ml) was calculated. Calibration curves were prepared by plotting this parameter against the known concentration of the drug in the range 30–4000 ng/ml. The line of best fit to the data points was obtained by the method of least squares.

Accuracy

The accuracy of the procedure was checked after replicate injections of the same concentration of $CMSOEN_2$ or after analysis at day intervals of different amounts of the drug added to pooled drug-free human blood.

Chromatography

A 150×2.1 mm I.D. stainless-steel column (Spherisorb 5-μm silica, HPLC Technology, Macclesfield, U.K.) was used. Analysis was performed with an 11-min linear gradient, starting from dichloromethane–ethanol (99.5:0.5) to dichloromethane–ethanol (95:5), and then the column was eluted isocratically with dichloromethane–ethanol (95:5) for 4 min. The mobile phase was delivered at a flow-rate of 0.4 ml/min and the column effluent was monitored at 230 nm (λ_{max} of $CMSOEN_2$).

RESULTS AND DISCUSSION

The extractability of $CMSOEN_2$ and the internal standard was measured in recovery experiments by using different solvents (chloroform, ethyl acetate, dichloromethane). The extraction yields were similar but we selected dichloromethane for its lower boiling point, which allows a rapid evaporation under nitrogen at 40°C, and for the good stability of the drug in this solvent. The extraction efficiency was slightly better for the internal standard (84.9±4.8%) than for $CMSOEN_2$ (81.8±5.1%) ($n=10$).

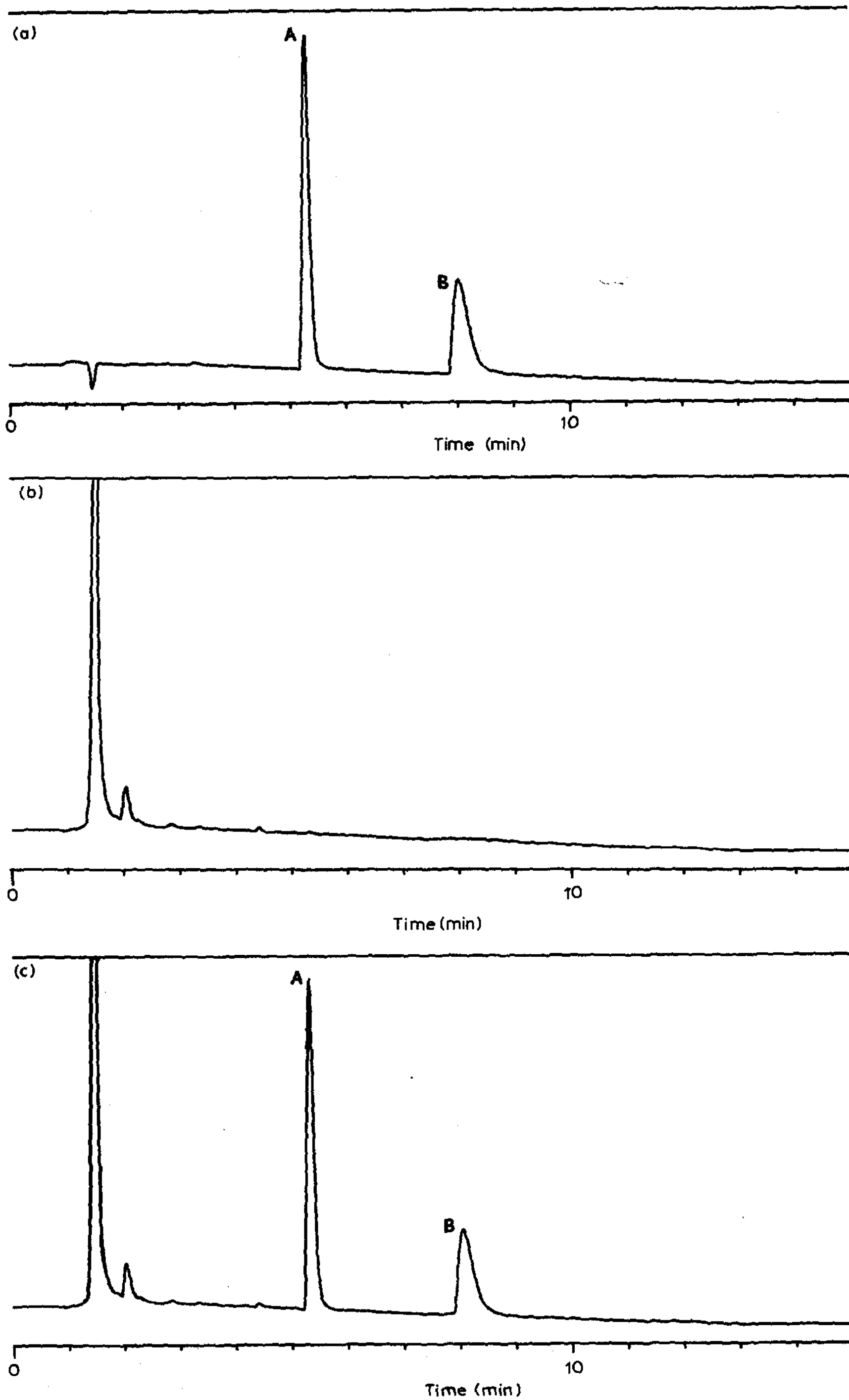


Fig. 2. Chromatograms of (a) a standard solution (internal standard and $CMSOEN_2$); (b) a blood extract obtained from a patient; (c) a blood extract obtained from the same patient treated with $CMSOEN_2$. Peaks: A = internal standard; B = $CMSOEN_2$.

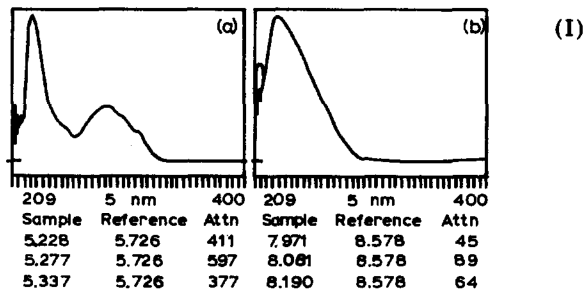
TABLE I

ACCURACY OF THE HPLC METHOD FOR THE DETERMINATION OF $CMSOEN_2$ IN BLOOD SAMPLES

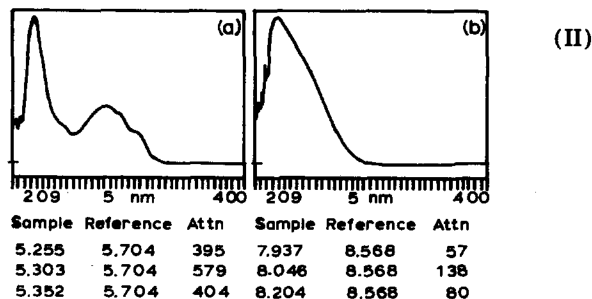
Theoretical concentration (ng/ml)	Observed concentration (mean ± S.D., $n=5$) (ng/ml)	C.V. (%)	Accuracy (%)
4000	3996 ± 394	9.9	99.9
2000	2007 ± 132	6.6	100.4
1000	983 ± 78	7.9	98.3
250	233 ± 20	8.6	93.2
62.5	60.6 ± 5.7	9.4	96.9

Calibration curves of peak-height ratios against concentrations of $CMSOEN_2$ were found to be linear in the concentration range used (30–4000 ng/ml). The equation of the linear regression line of best fit was $y=7.075x-3.897$ where y = $CMSOEN_2$ concentration (ng/ml) and x = (drug peak height/internal standard peak height) multiplied by the added concentration of internal standard (ng/ml). The correlation coefficient, 0.9999, shows the excellent linearity of the calibration curve in the concentration range used.

Despite the low molar extinction coefficient ($\epsilon_{max}=9000$) of $CMSOEN_2$, the



(I)



(II)

Fig. 3. (I) UV spectral analysis (200–400 nm) of a standard solution containing the two reference compounds: internal standard (a) and $CMSOEN_2$ (b). (II) UV spectral analysis (200–400 nm) monitored from a blood extract of patient treated with $CMSOEN_2$. (a) Internal standard; (b) $CMSOEN_2$.

TABLE II

PHARMACOKINETIC PARAMETERS FOR THREE PATIENTS AFTER INTRAVENOUS PERFUSION OF $CMSOEN_2$

Perfusion time, 15 min; $CMSOEN_2$ concentration, 45 mg/m^2; AUC = area under blood concentration–time curve from time 0 to infinity; $t_{1/2\beta}$ = elimination phase half-life; V_d = distribution volume; Cl = clearance rate.

Subject No.	Dose (mg)	AUC (μg/h/ml)	$t_{1/2\beta}$ (min)	V_d (l)	Cl (l/h)
1	90	0.9	24	52	90
2	85	1.16	41	72	73
3	68	1.01	37	59	67
Mean ± S.D.		1.05 ± 0.09	34 ± 8.9	61 ± 10.1	77 ± 11.9

detection limit was 30 ng/ml. The coefficient of variation (C.V. = 3.8%) obtained after replicate injections (n = 8) at this concentration shows the good reproductibility of the chromatographic system. The day-to-day precision of the assay also demonstrated little variability, with C.V. < 10% for each concentration tested (Table I).

Chromatograms of standards, drug-free blood and blood from a patient treated with a 15-min perfusion of $CMSOEN_2$ (Fig. 2) show distinct, well resolved peaks for the drug, the internal standard and endogenous components of blood. The retention times were 5.3 min for internal standard and 8.0 min for $CMSOEN_2$. UV spectral analysis shows the excellent purity of the two compounds, with a perfect superimposition of the three monitored spectra (upslope, apex and downslope) (Fig. 3).

The method was applied to the analysis of blood samples obtained from three patients after a 15-min perfusion of $CMSOEN_2$ (45 mg/m^2). The blood disappearance of $CMSOEN_2$ was a first-order kinetic process. The pharmacokinetic parameters are summarized in Table II. The values of the areas under the curve were comparable for the three patients. However, the elimination half-life of subject 1 was shorter (24 min) than that of the two other subjects (41 and 37 min). An important factor to be considered in the proposed method is the direct extraction of the drug from whole blood instead from plasma, which avoids a centrifugation step and the possible degradation of the drug during this step. Such a procedure can be useful for the assay of drugs that are unstable in aqueous media, such as nitrosoureas.

Since the apparatus used provides useful information on the purity and the identity of the chromatographic peaks, the present assay method is safe, rapid and sufficiently sensitive to allow an accurate determination of the pharmacokinetic parameters in patients now entered in phase II clinical trials.

ACKNOWLEDGEMENTS

The authors thank Mrs. J. Lefrancois and D. Montjotin for the preparation of the manuscript.

REFERENCES

1 V.T. De Vita, P.P. Carbone, A.H. Owens, G.L. Gold, J.J. Krant and J. Edmonson, Cancer Res., 25 (1965) 1876.
2 T.H. Wasserman, M. Slavik and S.K. Carter, Cancer Treat. Rev., 1 (1974) 131.
3 T.H. Wasserman, M. Slavik and S.K. Carter, Cancer Treat. Rev., 1 (1974) 251.
4 J. Oiry and J.L. Imbach, Eur. J. Med. Chem., 19 (1984) 305.
5 R. Maral, C. Bourut, E. Chenu, G. Mathé, J. Oiry and J.L. Imbach, Eur. J. Med. Chem., 19 (1984) 315.
6 D. Godeneche, J.C. Madelmont, M.F. Moreau, J. Duprat, J.L. Chabard, R. Plagne and G. Meyniel, Drug Metab. Dispos., 13 (1985) 220.
7 D. Godeneche, J.C. Madelmont, M.F. Moreau, J. Duprat, R. Plagne and G. Meyniel, Drug Metab. Dispos., 14 (1986) 112.
8 J.C. Madelmont, D. Godeneche, J. Oiry, J.L. Imbach, M.F. Moreau, D. Parry and G. Meyniel, Brevet International No. 85 90 1474-8, Nov. 26, 1985.
9 J.C. Madelmont, D. Godeneche, D. Parry, J. Duprat, J.L. Chabard, R. Plagne and G. Meyniel, J. Med. Chem., 28 (1985) 1346.
10 C. Bourut, E. Chenu, D. Godeneche, J.C. Madelmont, R. Maral, G. Mathé and G. Meyniel, Br. J. Pharmacol., 89 (1986) 539.
11 D. Godeneche, J.C. Madelmont, P. Labarre, R. Plagne and G. Meyniel, Xenobiotica, 17 (1987) 59.
12 V.A. Levin, W. Hoffman and R.J. Wienkman, Cancer Treat. Rep., 62 (1978) 1305.
13 R. Lemoine and A. Gouyette, Cancer Treat. Rep., 63 (1979) 1335.
14 V.A. Levin, J. Liu and R.J. Weinkman, Cancer Res., 41 (1981) 3475.
15 J.A. Montgomery, R. James, G.S. McCaleb and T.P. Johnston, J. Med. Chem., 10 (1967) 668.