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LC determination of a sulphur mustard decontaminant CC-2 in rat serum☆

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the analysis of *N*,*N*′-Dichlorobis (2,4,6-trichlorophenyl) urea (CC-2), a potent sulphur mustard decontaminant, in rat serum. The HPLC analysis, applicable to 0.5 ml volumes of serum, involved double extraction of serum samples with diethyl ether at alkaline pH followed by separation on a RP-18 column and the use of UV detector at 230 nm. The method was sensitive with a limit of quantitation of 10 ng ml^{-1} in rat serum and the recovery was always $>90\%$. Excellent linear relationships ($r > 0.99$) were obtained between the measured and added concentration ratios of the serum concentrations over a range of 10–200 ng ml^{-1}. The precision and accuracy were acceptable as indicated by relative standard deviation ranging from 2.47 to 17.49%, bias values ranging from -4.35 to 13.21%. Moreover, CC-2 was found stable in rat serum even after 3 months of storage at -60 °C and being subjected to three freeze–thaw cycles. The assay was found to be sensitive, specific, accurate, precise, and reliable for use in pharmacokinetic studies.

Keywords: Reversed phase chromatography; CC-2; Sulphur mustard decontaminant; Serum

1. Introduction

In the quest for specific antidotes for frequently used warfare agent (sulphur mustard or mustard gas) toxicity, decontamination formulations containing chloramines were prepared at Defence Research & Development Establishment (DRDE), Gwalior, India. *N*,*N*′-Dichlorobis (2,4,6-trichlorophenyl) urea (CC-2)-based formulations (Fig. 1) exhibited higher stability and efficacy [1,2]. Till date, there is no published report on either high-performance liquid chromatographic (HPLC) assay method or pharmacokinetics of CC-2. Therefore, an HPLC assay method has been developed and validated for the quantitative estimation of CC-2 in rat serum, which was applied to study its pharmacokinetics in rats.

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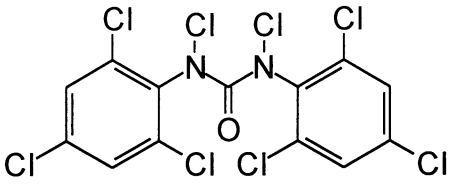

Fig. 1. Chemical structure of CC-2.

2. Experimental

2.1. Materials

Compound CC-2 (purity > 99%) was provided by DRDE and was used in the present study. HPLC grade acetonitrile was purchased from J.T. Baker, USA. HPLC grade dichloromethane and methanol were purchased from Ranbaxy Laboratories, SAS Nagar, India. Analytical grade di-potassium hydrogen orthophosphate, potassium dihydrogen orthophosphate and orthophosphoric acid were procured from Qualigens Fine Chemicals, Bombay, India. Diethyl ether was further purified by distillation before use. Triple distilled water from all Quartz glass apparatus was used in the preparation of the buffer.

Blood was collected from healthy male Sprague–Dawley rats and was centrifuged to separate serum so as to generate a drug-free serum pool.

2.2. Instrumentation

A binary HPLC pump (LC-10AD with SCL-10A VP system controller, Shimadzu, Japan) was used to pump the mobile phase (pump A: 20 mM potassium dihydrogen orthophosphate buffer, pH 3.5 and pump B: 65% acetonitrile in 20 mM potassium dihydrogen orthophosphate buffer, pH 3.5) at a flow rate of 1 ml min^{-1}. A Model 7125i syringe loading injector (Rheodyne, USA) fitted with a fixed 50 μl loop was used to inject the samples. Separation was achieved on a RP-18 column (5 μm, 100 × 4.6 mm, id), coupled with a guard column packed with the same material (5 μm, 30 × 4.6 mm, id) (Pierce, Rockford, IL). The eluants were monitored using a UV detector (Gynkotek, FRG) set at 230 nm and chromatograms were integrated using Class-VP software (Shimadzu, Japan). The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml min^{-1} before analysis commenced. A vortex-mixer (Cecon, India), ultrasonic bath (Bransonic, Shelto, CY), a Model SVC-220H Speed vac concentrator (Savant, NY) and a Model C-30 centrifuge (Remi, India) were used for sample preparations.

2.3. Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile and potassium dihydrogen orthophosphate buffer (pH 3.5) (55:45, v/v) and mixed well. The mobile phase was degassed for 15 min before use and was pumped at a flow rate of 1 ml min^{-1}. The chromatography was performed at ambient temperature.

2.4. Stock and standard solutions

A 100 μg ml^{-1} stock solution of CC-2 was prepared by dissolving 5 mg of the compound in minimum volume of dichloromethane and making up the volume to 50 ml with methanol and was used to prepare working standards containing 0.4, 0.8, 2, 4 and 8 μg ml^{-1} of CC-2 in methanol. Analytical standards (50, 100, 250, 500 and 1000 ng ml^{-1}) were prepared by diluting each 625 μl of the corresponding working standards with 5 ml of reconstitution solution (55% acetonitrile in 20 mM di-potassium hydrogen orthophosphate buffer (pH 7.0)).

Serum calibration standards containing 10, 20, 50, 100 and 200 ng ml^{-1} of CC-2 were prepared individually by diluting 25 μl of the working standards to 1 ml with serum. This method was used to prepare appropriate standards in serum in replicate.

2.5. Extraction procedure

Blank or spiked serum (0.5 ml) was basified with 50 μl of 1M potassium hydroxide. To this, 2 × 3 ml diethyl ether was added, vortex-mixed for 1 min and centrifuged at 1000 × g for 10 min. The supernatant was transferred to a 10 ml coni-

cal tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness in Savant speed vac concentrator. The residue was reconstituted in 0.1 ml reconstitution solution, centrifuged and the resulting solution was injected onto the HPLC system. The external standard method was used for quantitation.

2.6. Recovery

CC-2 was added to serum at three concentration levels. Aliquots (0.5 ml) of serum spiked at 20, 50, and 200 ng ml^{-1} of CC-2 were processed as described above and the concentrations were determined from the regression of the analytical standard calibration curve. Recovery was calculated by comparing the observed concentrations with the spiked concentrations.

2.7. Accuracy and precision

Accuracy and precision of the assay method were studied in spiked serum samples at low (20 ng ml^{-1}), medium (100 ng ml^{-1}), and high concentrations (200 ng ml^{-1}) of CC-2. One set of the samples consisting of low, medium and high concentrations was assayed in triplicate on the day of preparation. The concentration of the compound in the samples were determined from the calibration curve constructed from the standards prepared by spiking the serum with CC-2 to their true or normal value. The assay was repeated with another set of samples spiked at the three concentrations on three more occasions. Intra- and inter-batch accuracy was determined by calculating the %bias from the theoretical concentration. Precision, in terms of relative standard deviation (RSD) was obtained by subjecting the data to one way analysis of variance (ANOVA).

2.8. Stability of CC-2 in spiked serum stored at −60 °C

Effect of storing the serum samples spiked with CC-2 at −60 °C on the stability of CC-2 was studied at low, medium and high concentration levels as mentioned above. Replicate serum samples at the three concentration levels were prepared and one set of the samples consisting of low, medium and high concentrations was assayed in triplicate as outlined above. The results of day 1 (analyzed on the day of preparation) were considered as 100% and the remaining samples were stored at −60 °C. These samples were assayed after 0.5, 1 and 3 months and the results were compared with the initial concentration, in terms of %deviation.

2.9. Effect of freeze–thaw cycles on the stability of CC-2

Drug-free serum was spiked at the low, medium and high concentrations. One set (comprising triplicates of each concentration level) was assayed on the day of preparation as described above with serum calibration standards, without being subjected to the freeze–thaw cycle and the results were referred as standard (100%). The remaining three sets of samples were stored frozen at −60 °C and were assayed after one, two and three freeze–thaw cycles and their concentrations were determined with the respective calibration curve. Thawing was achieved by keeping the tubes containing the sample at room temperature for 30 min. Data was analyzed as described for the stability of CC-2 in spiked serum stored at −60 °C.

2.10. Pharmacokinetic analysis

A suspension formulation of CC-2 (1.0 g ml^{-1}) was prepared using 1% gum acacia and the rats were fed orally with approximately 2.0 ml kg^{-1} of the formulation for pharmacokinetic evaluation. Blood samples were collected between 0 and 24 h after dosing, centrifuged and serum stored at −60 °C pending analysis. Serum samples were assayed for CC-2 and the concentration–time data was used for pharmacokinetic analysis using model-independent method [3]. The maximum serum concentration (C_{max}) and its time of occurrence (t_{max}) are the observed values. The area under the serum concentration–time curve from time zero to the time of final measurable sample (AUC_{0-t}) was calculated using the linear trapezoidal method.

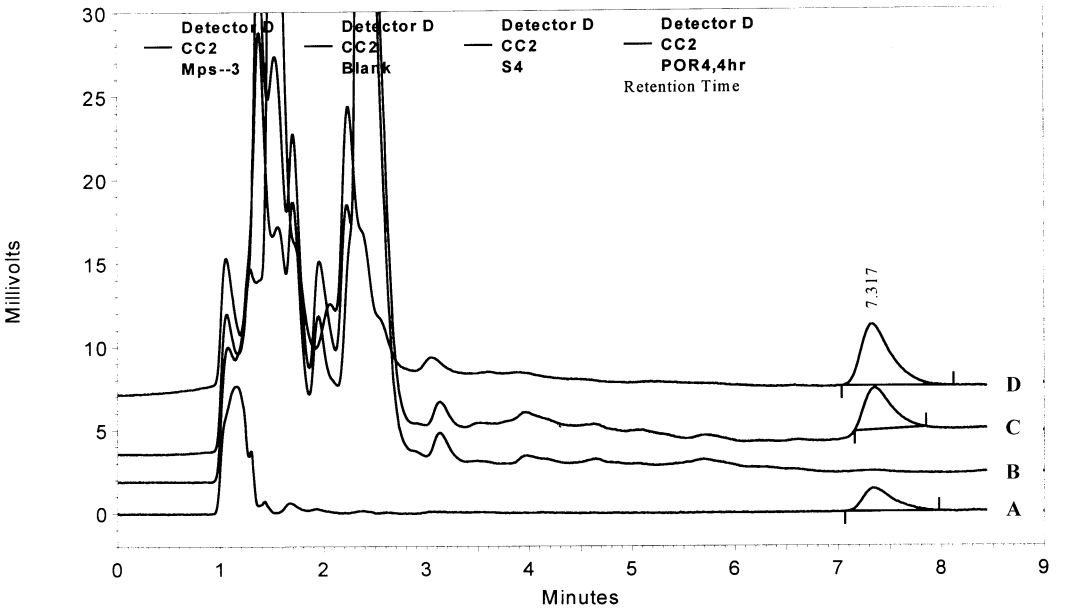

Fig. 2. Chromatograms of (A) standard containing 250 ng ml^{-1} CC-2, (B) drug-free rat serum; (C) serum containing 100 ng ml^{-1} CC-2; (D) rat sample taken 4 h after 2 g kg^{-1} oral dose of CC-2.

3. Results and discussion

The compound is poorly soluble in methanol and highly soluble in dichloromethane and acetonitrile. But, it is unstable in acetonitrile as it develops yellow colour and decrease in peak response. Therefore, the compound was dissolved in minimum volume of dichloromethane followed by addition of desired volume of methanol.

Initially, the UV spectrum of CC-2 was determined which showed a maximum absorbance (λ_{max}) at 230 nm. Detection wavelength and pH of the phosphate buffer of the mobile phase were optimized by comparing UV absorbance of CC-2 using a solution containing 50 μg ml^{-1} CC-2 in a 1:1 mixture of acetonitrile and phosphate buffer (20 mM; pH: 3.5, 4.0, 4.5, 5.0 and 6.0). There was no significant difference in the absorbance of CC-2 and hence, detection wavelength of 230 nm and mobile phase of pH 3.5 was used for validation. Replacement of the salt with ammonium acetate worsened the peak response of the compound. Among the various modifications tried, 55% acetonitrile in 20 mM phosphate buffer (pH 3.5) at a flow rate of 1 ml min^{-1} and the use of a RP-18 column (100×4.6 mm, 5μm) eluted the compound at optimum t_R (7.5 ± 0.3 min). The HPLC analysis showed that the lowest limit of quantitation was 10 ng ml^{-1} of CC-2 in serum, which provided adequate sensitivity to conduct pharmacokinetic study in animals. Typical chromatograms of an analytical standard containing 250 ng ml^{-1} CC-2 (A); an extract of the drug-free rat serum (B); serum containing 100 ng ml^{-1} CC-2 (C); and rat serum sample taken 4 h after 2 g kg^{-1} oral dose are depicted in Fig. 2. Moreover, the extraction procedure and the chromatographic conditions yielded a clean chromatogram for the compound. The endogenous impurities did not interfere the elution of the compound, indicating that the method was selective.

A validation protocol was prepared and all criteria commonly employed during the validation of the HPLC methods were assessed. The method was validated in terms of reproducibility, linearity, recovery, accuracy and precision and stability studies of the compound in spiked serum samples stored at -60 °C and during freeze–thaw cycles.

HPLC system reproducibility was checked with pentaplet injections of each analytical standard in

a single run. The variations in the peak heights of each standard was maximum (5.28%) at 0.05 μg ml^{-1} and was $<1.6\%$ at other concentration levels indicating that the system yields reproducible data. Moreover, the peak heights were linear with the concentrations.

The recoveries of the compound from the spiked serum samples were calculated at three concentration levels (20, 50, and 200 ng ml^{-1}) of CC-2 and ranged from 91.19 to 107.88% with a CV of $<10.19\%$ (Table 1), thus inclusion of an internal standard was not deemed necessary. A linear relationship existed between peak heights and concentrations of CC-2 over a concentration range of 10–500 ng ml^{-1}. Linear regression analysis of standard curves yielded correlation coefficients all exceeding 0.99.

Table 1
Recovery of CC-2 in rat serum

Concentration (ng ml^{-1})		Recovery (%)	CV (%)
Theoretical	Observed		
20	18.24 ± 0.63	91.19	3.34
50	53.94 ± 5.56	107.88	10.19
200	191.93 ± 13.50	95.97	7.50

Table 2
Intra- and inter-assay accuracy and precision of CC-2 in rat serum

CC-2 concentration	Spiked sample		
	Low	Medium	High
Theoretical	20 ng ml^{-1}	100 ng ml^{-1}	200 ng ml^{-1}
Observed (Mean ± SD)	20.83 ± 3.73	95.46 ± 4.57	196.53 ± 20.21
%Bias$_{\text{intra-assay}}$	4.62	−4.35	−2.09
%Bias$_{\text{inter-assay}}$	13.21	−2.04	−1.28
RSD$_{\text{intra-assay}}$ (%)	12.53	5.34	11.37
RSD$_{\text{inter-assay}}$ (%)	17.49	2.47	5.96

Intra- and inter-batch accuracy and precision were assessed at CC-2 concentrations of 20, 100 and 200 ng ml^{-1} in replicates on 3 days. Three samples were analyzed at each concentration on the same day and the observed concentrations of the spiked control samples estimated from the calibration curve were compared to their true values. To determine the variance in precision (%RSD), the observed concentrations at each level were subjected to one way ANOVA and the RSD was calculated using the mean square value. An acceptance limit of 20% was employed for the low concentrations and 15% was applied for medium and high samples [4]. The results showed satisfactory intra-day precision and accuracy as indicated by RSD $\leqslant 12.53\%$ and bias of -4.35 to 4.62% (Table 2). Inter-day accuracy and precision were evaluated at the same concentrations as above and the results demonstrated satisfactory inter-day precision and accuracy as shown by RSD and bias values of $\leqslant 17.49\%$ and -2.04 to 13.21%, respectively.

Long-term stability of CC-2 in serum was evaluated following 90 days of storage at -60 °C at the low, medium and high concentration. The results of the first day analysis were considered as reference and the subsequent results were compared with the first day result by ANOVA. The day-to-day variation (%CV) was $\leqslant 9.44\%$ and changes (%bias) from the nominal concentrations ranged from -13.67 to 13.85% demonstrating that CC-2 was stable in serum for at least 90 days (Fig. 3A). The stability of CC-2 in serum was performed through three freeze–thaw cycles at the above mentioned concentrations. Thawing was achieved by keeping the samples at room temperature for 30 min. The day-to-day variation (%CV) was $\leqslant 12.23\%$. After three cycles, the % deviation from the concentration observed on day 1 were -17.04, -7.78 and -3.41% for the low, medium and high concentrations, respectively, indicating that the compound was stable in serum through three freeze–thaw cycles (Fig. 3B). It should be noted that these variations in the stability studies represent both stability parameter and inherent intra-/inter-batch variations. These observed variations were comparable to intra-/inter-batch precision. No trend

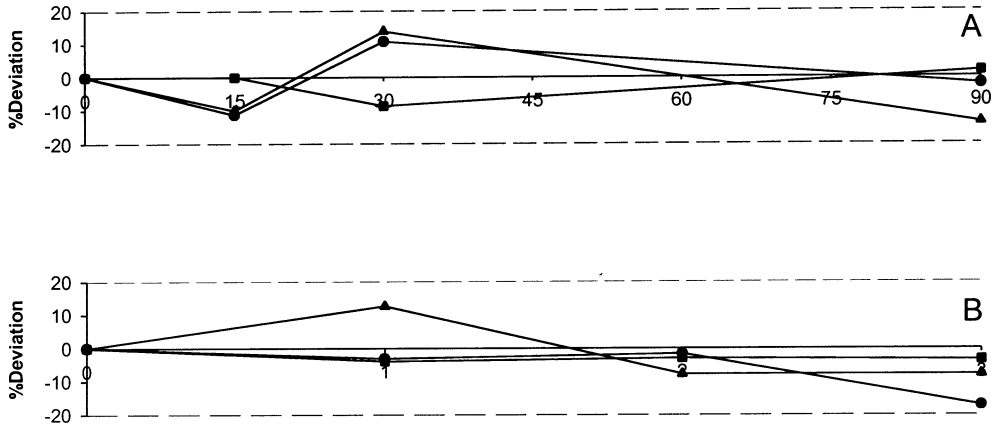

Fig. 3. Stability of CC-2 in serum (A) on storage at −60 °C; and (B) during freeze–thaw cycle at low (●, 20 ng ml^{-1}), medium (▲, 50 ng ml^{-1}) and high (■, 200 ng ml^{-1}).

was observed in the read concentrations either after 90 days of storage at −60 °C or three freeze–thaw cycles.

3.1. Application of the method in preclinical pharmacokinetics

The assay described here was applied to characterize the pharmacokinetic profile of CC-2 in rat following 2 g kg^{-1} oral administration of a suspension formulation of CC-2. CC-2 was quantifiable in serum between 0.5 and 24 h after oral dose (Fig. 4) and exhibited two C_{max} values. The mean C_{max} of CC-2 were 248.7 ± 37.9 ng ml^{-1} ($C_{max,1}$) and 129.5 ± 0.9 ng ml^{-1} ($C_{max,2}$) which were attained after 1.0 and 8.0 h of dosing. The AUC_{0-24h} was 1765 ng h ml^{-1} (Table 3).

4. Conclusions

An HPLC assay was developed and validated for the quantitative determination of CC-2 in rat serum. The extraction procedure is easy. The moderate analysis time, together with rapid eva-

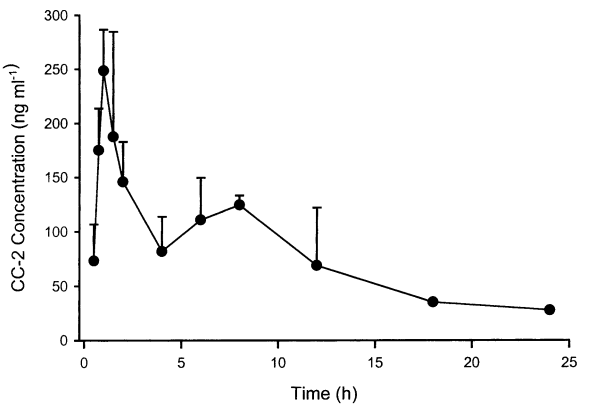

Fig. 4. Mean ($n = 3$) serum concentration–time profile of CC-2 in rats after 2 g kg^{-1} oral dose.

Table 3
Pharmacokinetic parameters of CC-2 in rats ($n = 3$) after 2 g kg^{-1} oral dose

Parameter (unit)	Mean ± SD
$C_{max,1}$ (ng ml^{-1})	248.7 ± 37.9
$C_{max,2}$ (ng ml^{-1})	129.5 ± 0.9
$t_{max,1}$ (h)	1.0
$t_{max,2}$ (h)	8.0
AUC_{0-t} (ng h ml^{-1})	1765

poration of extraction solvent, allowed rapid analysis. The method was shown to be accurate and reliable over a concentration range of 10–500 ng ml^{-1} with an acceptable RSD and bias. The method is now in routine use in our laboratory.

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