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ANALYSIS OF CARMINOMYCIN IN HUMAN SERUM BY FLUOROMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is given for the determination of carminomycin (CMM) and a major metabolite carminomycinol (CMMOH) in serum from cancer patients after intravenous administration of carminomycin as the free drug.

CMM and CMMOH are extracted from serum with chloroform, the extract evaporated and the residue dissolved in methanol. High-performance liquid chromatography analysis utilized a C_{18} μBondapak reversed-phase column eluted with 0.1 mol/l acetate buffer (pH 4) — acetonitrile (60:40, v/v) with fluorescence detection. The assay is linear, reproducible, and precise with a limit of detection of 2 ng/ml. Representative serum levels of CMM and CMMOH in a cancer patient are presented.

INTRODUCTION

Carminomycin · HCl ($C_{26}H_{27}NO_{10}$ · HCl, free base MW 513.51, NSC-180024) is an anthracycline antibiotic with antineoplastic activity. It is isolated from the mycelia of *Actinomadura carminata* [1, 2]. The structure of carminomycin (CMM) (Fig. 1) is shown in relationship to the structures of adriamycin and daunomycin. All three compounds contain the amino sugar daunosamine and differ slightly at carbons 4 and 14. CMM is metabolized by reduction

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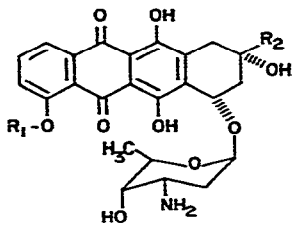

	R_1	R_2
ADRIAMYCIN	CH_3	$COCH_2OH$
ADRIAMYCINOL	CH_3	$CH(OH)CH_2OH$
DAUNOMYCIN	CH_3	$COCH_3$
CARMINOMYCIN	H	$COCH_3$
CARMINOMYCINOL	H	$CH(OH)CH_3$

Fig. 1. Structures of adriamycin (ADM) and metabolite adriamycinol, daunomycin, and carminomycin (CMM) and metabolite carminomycinol (CMMOH).

of the ketone at C_{13} to an alcohol, carminomycinol (CMMOH), a major metabolite. CMMOH has cytotoxic activity comparable with CMM at approximately equivalent optimal doses and similar maximum percentage increase in median survival times of treated vs. control mice on two different dose schedules [3, 4].

Although CMM is chemically similar to adriamycin (ADM), recent studies suggest it may have a mechanism of action different from ADM [5]. CMM is being considered as an alternative to ADM chemotherapy due to its reported lower incidence of cardiotoxicity [6]. Reports have been published on the analysis and kinetics of ADM and metabolites in human serum [7—9], but not on the analysis and kinetics of CMM and its metabolites.

This paper gives an analytical method for simultaneous determination of CMM and CMMOH in serum following intravenous injection of CMM as the free drug from the first dose of therapy. The development of the assay was based on the extraction properties and reversed-phase high-performance liquid chromatographic (HPLC) separation of other anthraquinone glycosides. The proposed assay includes extraction from the buffered serum sample into an organic phase, re-extraction into a slightly acidic aqueous phase and separation by reversed-phase HPLC. A high detection sensitivity and selectivity of the assay is achieved by using fluorescence detection.

EXPERIMENTAL

Drugs and reagents

Carminomycin 1 (Lot 78F581, 95% by assay) and CMMOH were from Bristol Labs. (Syracuse, NY, U.S.A.). ADM·HCl (batch No. 565), manufactured by Farmitalia (Milan, Italy), and outdated human serum, used for the standards, were supplied by SUNY/Upstate Medical Center (Syracuse, NY, U.S.A.). Clinical chemistry control serum was purchased from General Diagnostics (Avon, CT, U.S.A.).

Methanol, hexane, chloroform, and acetonitrile were of spectroquality Burdick and Jackson glass-distilled grade (Rainin Instrument, Brighton, MA, U.S.A.). Glacial acetic acid, monobasic potassium phosphate, sodium hydroxide solution, and ammonium hydroxide were of certified ACS grade. Deionized water filtered through a Millipore® apparatus was used throughout.

Preparation of stock aliquots

Ten mg of each drug were dissolved in 5 ml of distilled water. The CMM and CMMOH solutions were combined and quantitatively diluted to 0.2 mg/ml for each compound. The ADM solution was similarly diluted. Aliquots (0.2 ml) of each solution were transferred to disposable polypropylene tubes and stored frozen at —20°C or colder.

Preparation of working serum standards

Spiked serum standards were prepared in 13 × 100 mm PTFE-lined screw cap test tubes as follows.

A stock aliquot of CMM and CMMOH was diluted 1:20 with methanol in a polycarbonate tube and 50 µl and 20 µl of this solution were pipetted, in duplicate, to 2.0 ml of serum. A sample of the 1:20 dilution was diluted 1:10 with methanol and 100, 50, 20, and 5 µl of the resulting solution pipetted, in duplicate, to 2.0 ml of serum. The additions to serum were carried out rapidly prior to the addition of ADM. This results in final concentrations of 250, 100, 50, 25, 10, 7.5, 5 and 2.5 ng CMM and CMMOH per ml each. Standards were run with each set of patient samples.

Assay extraction procedure

A stock aliquot of ADM, the internal standard, was diluted 1:200 with methanol and 100 µl of the dilution were added to each 2-ml serum standard and 2-ml patient's clinical serum sample to give 50 ng ADM per ml. To each

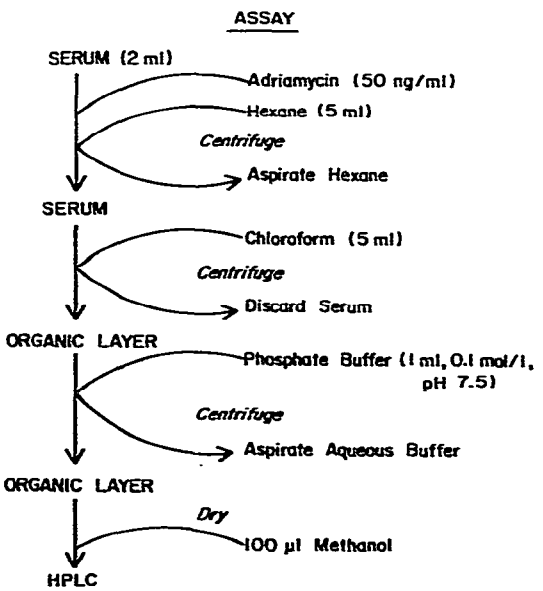

Fig. 2. Schematic flow diagram of the CMM assay in human serum.

standard and sample were added 5.0 ml of hexane. Each capped tube was shaken for 5 min on a rotorack at 35 oscillations per min and centrifuged at 2600 *g* for 15 min at ambient temperature. The hexane layers were aspirated and discarded. To each aqueous layer, 5 ml of chloroform were added; and each tube was capped, shaken, and centrifuged as before. The serum layer was aspirated and discarded. One ml of 0.1 mol/l phosphate buffer (pH 7.5), was added to each tube to wash the chloroform. The aqueous buffer was aspirated and discarded following mixing and centrifugation. Each extract was evaporated to dryness under a stream of filtered nitrogen in a water bath at 37°C, kept at ambient temperature overnight and reconstituted in 100 μl methanol, 80 μl of which were chromatographed. Fig. 2 is a schematic flow diagram of the assay.

High-performance liquid chromatography parameters

The HPLC system consisted of a U6K injector with a 2-ml loop (Waters Assoc., Milford, MA, U.S.A.), an Altex Model 110 pump (Rainin Instrument), and a Spectra Glo Fluorometer (No. 3301, Gilson Medical Electronics, Middletown, WI, U.S.A.). Fluorometric detection utilized a 15-μl glass flow-cell assembly (No. 3382) equipped with interference filters at 380—480 nm for excitation (No. 5-60) and an emission filter with a lower cut-off of 560 nm (No. 3-66). A scan of the intrinsic fluorescence spectrum of CMM, CMMOH, and ADM using a spectrophotofluorometer (Aminco-Bowman, American Instruments, Silver Springs, MD, U.S.A.) showed an excitation maximum of ca. 470 nm and an emission maximum at 545—550 nm for all three compounds. The column was a Waters Assoc. C_{18} μBondapak reversed-phase type (30 cm × 3.9 mm I.D., 10-μm particle size, used at ambient temperature. The mobile phase was 0.1 mol/l ammonium acetate buffer (pH 4.0)—acetonitrile (60:40, v/v). The buffer first was filtered through a 0.45-μm Millipore® filter and the eluent was de-aerated in a sonic bath. The column was conditioned with the mobile phase for approximately 1—2 h before use. The flow-rate was 1.9 ml/min, at a column head pressure of 130—150 bar with the mobile phase recycled.

Quantitation

Detector output was displayed on a Hewlett-Packard recorder Model 7123A and collected and processed by a Hewlett-Packard Model 3354/C Laboratory Automation System. Each chromatogram was interpreted using the Model 3354/C software system. The chromatographic data for each chromatogram were collected for each set of analyses and stored on a disc. The retention time, maximum signal and integral (signal × time) for CMM, CMMOH, and ADM were automatically obtained for each chromatogram. At the end of each series of analyses, the regression of peak height ratio (CMM or CMMOH response/ADM response) versus CMM or CMMOH concentration in the spiked standards was calculated by least-squares analysis and the concentration of CMM or CMMOH in each serum sample was estimated by inverse prediction [10].

RESULTS AND DISCUSSION

Chromatography

In preliminary work, reversed-phase chromatography on C_{18} and alkylphenyl μBondapak columns was compared with normal-phase adsorption chromatography on a microparticulate silica column, namely, Partisil 10 PAC PXS 10/25 (Whatman, Clifton, NJ, U.S.A.). Normal-phase chromatography had variable and skewed peak heights and variable retention times. In general, normal-phase columns can become deactivated by the extent of hydration from solvents. Also noted was loss of peak height when drug residues were reconstituted in the normal-phase eluent [chloroform—methanol—acetic acid—water (80:20:2:3)] and allowed to stand 1 h at room temperature. For the drugs studied here, reversed-phase was less sensitive to small changes in solvent composition. Our results correspond with recent publications recommending reversed-phase HPLC for ADM and metabolites [11], although normal-phase chromatography has been used for CMM measurement [12]. The results using a reversed-mode C_{18} column for the separation of CMM, CMMOH, and ADM showed improved resolution, recovery, linearity, and reproducibility over normal-phase HPLC.

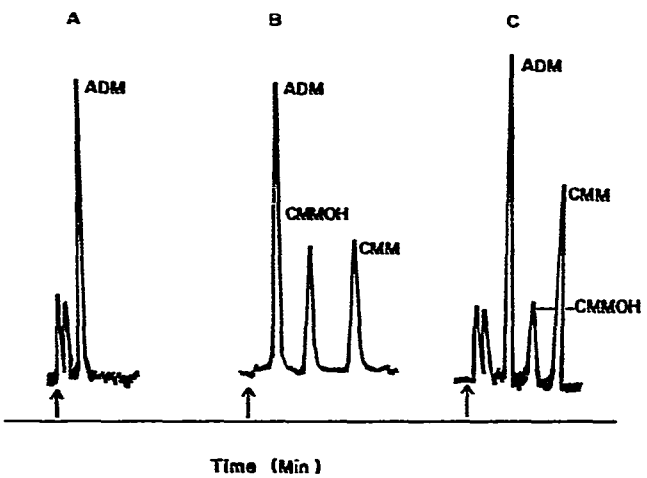

Fig. 3. (A) Elution profile of ADM, the internal standard of 50 ng/ml, extracted from a zero time patient serum sample. (B) Elution profile of serum supplemented with 25 ng/ml CMM and 25 ng/ml CMMOH with internal standard 50 ng/ml ADM. (C) Elution profile of serum sample from a patient administered 22 mg/m² CMM 30 min previously. Peaks represent: 50 ng/ml ADM, 14.16 ng/ml CMMOH and 29.5 ng/ml CMM.

ADM was chosen as the internal standard because of its similar structure and chromatographic behavior, enabling a short analysis time of 7 min per sample. Typical chromatograms, including spiked samples and a clinical sample, are shown in Fig. 3. Consistent retention times of 2.6 min for ADM, 4.0 min for CMM, and 5.8 min for CMMOH were obtained.

The composition of the mobile phase was influenced by CMM being a weak difunctional acid with a pK_{a1} of 8.00, requiring an acid modified in an organic solvent to reduce the elution time from a C_{18} column. After a day's

run, the column was flushed with acetonitrile—water (70:30, v/v) to avoid column degradation by long-term exposure to acidic pH.

Assay procedures

Water, methanolic HCl, chloroform—methanol—acetic acid—water (80:20:2:3, v/v), acetate buffer (pH 4.0)—acetonitrile (60:40, v/v), and methanol were examined as reconstitution solvents. The results (Table I) indicated that methanol was superior. The buffer wash at the final assay step was examined at pH 6.5, 7.5, and 8.5 without any significant differences in recovery. Schoeffel (Model SF970) and Waters fluorometers were compared in series to the Gilson fluorometer, but provided equivalent or less sensitivity than the Gilson. A commercial control serum was analyzed to check that interfering endogenous substances were excluded in the assay. The aglycone metabolites of CMM were not studied or quantified here. Typically, sample preparation requires 3—4 h of operator time for 40 samples, and 7—8 h for instrumental analysis. The reproducibility and stability of multiple CMM and ADM frozen aliquots were constant (Table II).

Linearity

The equations of the line of best fit for typical calibration curves for the CMM and CMMOH standards were $y=0.017X + 0.015$, and $y=0.020X - 0.008$,

TABLE I

STABILITY OF CARMINOMYCIN IN VARIOUS SOLVENTS

Concentration of carminomycin 1 ng/μl.

Reconstitution solvent	Area (K)	Time (h)	
100% Water	55.9	0	
	36.5	1	
	31.1	2	
	21.7	4	
	9.5	24	
Chloroform—methanol—acetic acid—water (80:20:2.3)	68.0	0	
	74.7	1	Separation into 2 phases
	69.9	2	
	71.6	4	
	68.9	24	
0.1 mol/l ammonium acetate (pH 4)—acetonitrile (60:40)	62.7	0	
	58.0	1	
	51.8	2	
	46.2	4	
	15.8	24	
100% Methanol	63.4	0	
	62.7	1	
	62.3	2	
	63.9	4	
	66.9	24	

TABLE II

CARMINOMYCIN AND ADRIAMYCIN ALIQUOTS

Aliquot	Injections	Average peak height			
		0 h	2 h	4 h	24 h
Carminomycin					
A	1	25.6	22.9	20.6	20.5
	2				
	3				
B	1	23.8	21.2	19.4	19.4
	2				
	3				
C	1	25.6	25.2	19.5	20.0
	2				
	3				
		0 h	2.5 h	5 h	24 h
Adriamycin					
A	1	32.9	29.9	30.5	33.7
	2				
	3				
B	1	32.0	33.4	29.3	33.0
	2				
	3				
C	1	34.1	31.4	36.3	33.5
	2				
	3				

respectively; the coefficient of correlation (r) for both regressions was 0.999. Within the range of 0—250 ng/ml no tendency of the calibration data to deviate from linearity was observed at either end of the range.

Limit of detection

Assuming that the mean minus three times the standard deviation is a reasonable limit for the extreme lower limit of response for spiked samples, only one blank response (Table III, No. 16) for CMM was greater than this limit for 2.5 ng/ml standards. All the CMMOH blank values fell below the limit for 2.5 ng/ml CMMOH standards. This separation of response indicates that the limit of detection for both compounds is slightly lower than 2.5 ng/ml serum at the 95% confidence level. Therefore, 2 ng/ml serum is a practical limit for the assay.

Precision and reproducibility

A set of 50 ng/ml spiked standards was prepared as soon as the assay was developed and subsets of five or six replicates assayed at intervals (Table IV). The precision within any subset was good, the mean coefficient of variation (C.V.) being 4.4%. However, the mean observed values were statistically different ($p \leqslant 0.05$) on each of four days during a month. The first day being extremely different from those following, probably can be laid to operator inexperience, but the remaining variability must be expected to be normal for this assay.

TABLE III

INSTRUMENTAL RESPONSE TO EXTRACTED BLANKS AND CARMINOMYCIN (CMM) AND CARMINOMYCINOL (CMMOH) STANDARDS NEAR THE LIMIT OF DETECTION

Solution No.	Peak height (arbitrary units)			
	4.0 min retention time; 2.5 ng CMM per ml serum	Blank serum	5.8 min retention time; 2.5 ng CMMOH per ml serum	Blank serum
1	772	0	698	0
2	498	0	459	0
3	689	181	632	0
4	661	146	546	0
5	503	0	629	0
6	722	196	587	101
7	606	0	493	0
8	615	0	391	0
9	967	0	633	0
10	901	0	559	0
11	790	0	510	135
12	772	0	537	0
13	522	237	474	0
14	864	173	577	0
15	744	236	625	0
16	765	593	567	0
17	779	192	484	0
18	705	0	499	82
19	987	184	699	0
Mean	730		558	
S.D.	142		82	
C.V. (%)	19		15	

TABLE IV

ASSAY PRECISION

Time (days)	Mean observed CMM concentration (ng/ml)	N*	S.D.	C.V. (%)
1	58.3	6	3.1	5.3
5	36.1	6	1.5	4.2
13	42.6	5	2.0	4.8
26	39.8	5	1.3	3.3

ANOVA table**

	Sum of squares	DF	MS	F	Pooled S.D.
Treatment	1701	3	567	135	
Error	75	18	4.19		
Total	177	21			2.05

*All means are significantly different based on ANOVA and Duncan's Multiple Range Test.

**DF = degrees of freedom; MS = mean square; F statistic value = treatment mean square/ error mean square.

TABLE V

REPRODUCIBILITY OF CARMINOMYCIN ASSAY OF HUMAN SERUM
Dose = 22 mg/m².

Time (h)	Run 1 CMM concn. (ng/ml)	Run 2 CMM concn. (ng/ml)	Run 2 CMMOH concn. (ng/ml)
0.25	78.0	79.4	28.04
0.5	34.4	29.5	14.16
1.0	26.2	22.7	14.27
2	19.7	16.1	12.05
4	9.1	8.0	10.95
6	6.3	3.4	9.68
8	4.6	2.8	8.72
12	—	2.2	11.07
24	2.0	1.0	8.98

Reproducibility was assessed by analyzing a patient's sample set twice, at a one-month interval, with consistent results (Table V). Chronologically, regression equations were $y = 0.015X - 0.003$ and $y = 0.017X + 0.015$. Varying intercepts probably account for the slight concentration differences.

Application to biological samples

This assay has been employed in analyzing clinical serum samples for CMM and CMMOH. A typical serum concentration profile of CMM and CMMOH in man is shown in Fig. 4. The patient received 22 mg of CMM per m² intravenously. The serum CMM decline appeared biphasic with an elimination phase half-life of 12.5 h. CMMOH was detected within 15 min. After an initial decrease, the CMMOH concentration did not significantly change over

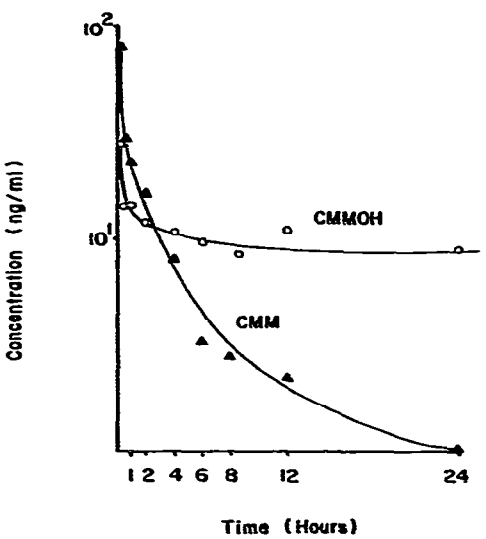

Fig. 4. Concentration vs. time curve of CMM and CMMOH after intravenous infusion of 22 mg/m² CMM from the same patient as in Fig. 3 and Table V. ▲ = CMM and o = CMMOH.

the 24-h collection period. The CMMOH concentration was higher than CMM from 4 h through 24 h. We have found different metabolite concentrations with CMM than reported pharmacokinetic values for ADM [13]. CMM kinetic results were similar to daunomycin metabolism [14].

These results show the applicability of the proposed assay to serum samples from patients administered therapeutic doses of CMM. The results also indicate a need to determine serum levels of CMM and especially of CMMOH for longer than 24 h after dosing. The pharmacokinetics of CMM and the activity of CMMOH suggest that CMMOH may be an important contributor to CMM administration and could be a useful drug itself.

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