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Note

High-performance liquid chromatography as an alternative to micro-biological measurements in the assay of tetracyclines

AVA RAY* and ROY HARRIS

Department of Chemistry, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG (U.K.)

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There has recently been a move to replace expensive biological assays by chemical assays, *e.g.* high-performance liquid chromatography (HPLC). This is the case for some antibiotics such as the tetracyclines[1]. It has already been shown that comparable results can be obtained by HPLC and bioassay for the measurement of oxytetracycline from various sources[2]. Having shown that this HPLC method[2] is acceptable for straightforward assays it is important to see if it is robust enough for assaying samples kept under extreme conditions. Degradation in the samples should be equally reflected by bioassay and HPLC.

This report describes the adaptations of the previous HPLC method mentioned above, *e.g.*, flow-rate, temperature and column packing, to look at tetracyclines kept at elevated temperatures as part of an accelerated degradation experiment[3,4] and assayed by HPLC and bioassay. The reliability of the HPLC assay was also tested by the comparison of data for the same samples assayed after a three year shorter storage period.

EXPERIMENTAL

The compounds assayed were seven different tetracycline standards stored in ampoules under nitrogen at +20°C, +37°C or +56°C: chlortetracycline, 2nd International Standard (IS) (CTC); oxytetracycline, 1st IS (OTC); tetracycline, 2nd IS (TC); demeclocycline, 1st International Reference Preparation (IRP) (DEM); methacycline, 1st IRP (MET); minocycline, 1st IRP (MIN) and doxycycline, 1st IRP (DOX).

Stock solutions of tetracyclines, with the exception of OTC, were prepared in HPLC-grade water (250 μg ml^{-1}) and were either used directly for HPLC analysis or diluted in phosphate buffer (pH 6.0) for use in the biological assays. OTC was made up in 5 ml of 0.1 M hydrochloric acid and diluted to 100 ml with HPLC-grade water.

Biological assays were carried out using *B. pumilis* NCTC 8241, following the method recommended by the British Pharmacopoeia[5].

Samples were assayed chemically using an adaptation of a previously reported HPLC method[2] adapted in our laboratory. The HPLC system consisted of a Spectra-Physics SP8100 liquid chromatograph equipped with an SP8110 autosampler and an

SP8440 variable-wavelength detector. An SP 4200 computing integrator was used to calculate peak areas and heights. Samples (2.5 μg in 10 μl) were chromatographed on a Spherisorb ODS-2 (5 μm) column (250 $\times$ 4.6 mm I.D.) (Phase Separations, Gwent, U.K.) maintained at 40°C and detected at 280 nm. The solvent system, 0.2 M ammonium oxalate–0.1 M NaEDTA–dimethylformamide (55:20:25, v/v/v) was degassed with helium and pumped at a flow-rate of 1 ml min^{-1} except in the cases of CTC and MIN where flow-rates of 1.2 ml min^{-1} and 1.5 ml min^{-1} respectively were used in order to reduce retention times. Each sample was chromatographed six times. Results from HPLC data were calculated as follows: (area of tetracycline peak in the sample/area of tetracycline peak in the standard) · labelled potency of standard and will be referred to hereafter as "potency" of sample notionally as IU mg^{-1}. Ratios were calculated for both types of assay relative to standard preparations stored continuously at -20°C.

RESULTS AND DISCUSSION

For most of the samples the HPLC and microbiological assay results were in good agreement. "Potency" values calculated for chemical and biological assays are shown in Table I. Since a linear relationship exists between tetracycline content measured by either area or height of the HPLC peak (see Fig. 1, correlation coefficient = 0.99), only integration values for areas were used in Table I. The relationship between HPLC and bioassay results are shown graphically in Fig. 2 which shows a correlation between the methods within acceptable limits (correlation coefficient = 0.96). The HPLC data were compared with HPLC results obtained for the same samples 3 years previously. When there was no degradation, the results are reproducible (Table II); where samples

TABLE I

COMPARISON OF "POTENCY" VALUES OBTAINED BY BIOASSAY AND HPLC

Average storage period = 20 years.

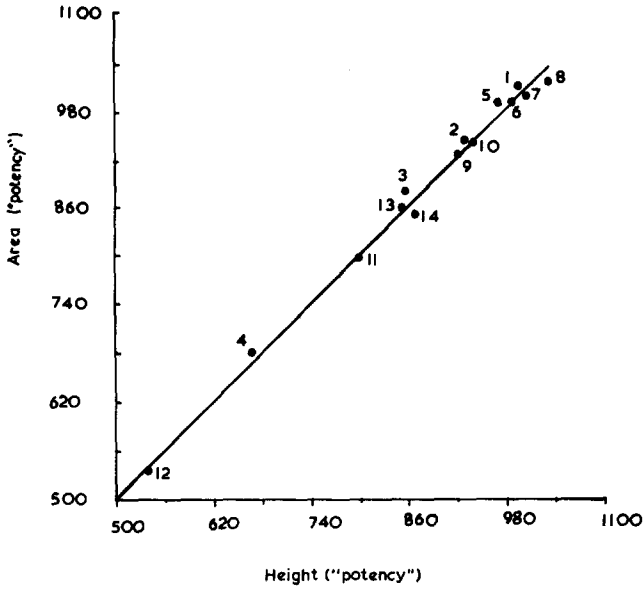| Compound (potency of standard IU mg^{-1})	Storage temperature (°C)	"Potency" mean as calculated by HPLC peak area (IU mg^{-1})	Bioassay (IU mg^{-1}) Mean (with fiducial limits)
CTC (1000)	20	1011.5	1022 (899.2–1164.2)
	37	943.8	889 (778.1–1009.8)
OTC (900)	20	880.9	828 (791.0–866.9)
	37	679.5	638 (607.3–670.1)
TC (982)	37	988.8	1007 (958.2–1058.5)
	56	990.3	983 (935.0–1032.9)
DEM (1000)	20	996.2	978 (939.8–1017.9)
	37	1016.5	1020 (980.0–1061.7)
MET (924)	37	928.0	952 (922.9–992.6)
	56	942.3	925 (892.1–959.3)
MIN (863)	37	797.2	868 (843.0–894.8)
	56	536.2	593 (579–608.7)
DOX (870)	37	854.5	856 (833.7–878.2)
	56	848.3	831 (809.6–853.0)

Fig. 1. Graph to show the linear relationship between "potency" means calculated by HPLC peak areas and peak heights for seven tetracyclines stored at elevated temperatures. CTC: 20°C(1), 37°C(2). OTC: 20°C(3), 37°C(4). TC: 37°C(5), 56°C(6). DEM: 20°C(7), 37°C(8). MET: 37°C(9), 56°C(10). MIN: 37°C(11), 56°C(12). DOX: 37°C(13), 56°C(14).
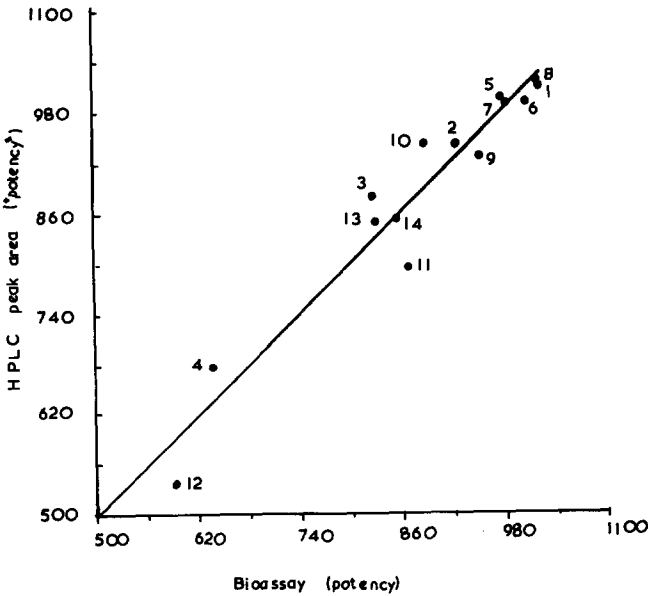

Fig. 2. Graph to show the comparison of "potency" values obtained by bioassay and those calculated from HPLC peak areas (see Table I). CTC: 20°C(1), 37°C(2). OTC: 20°C(3), 37°C(4). TC: 37°C(5), 56°C(6). DEM: 20°C(7), 37°C(8). MET: 37°C(9), 56°C(10). MIN: 37°C(11), 56°C(12). DOX: 37°C(13), 56°C(14).

TABLE II

COMPARISON OF "POTENCY" VALUES CALCULATED FROM HPLC DATA OBTAINED FROM THE PRESENT EXPERIMENT AND FROM HPLC ANALYSIS CARRIED OUT IN 1985 USING THE SAME SAMPLES

Sample (potency of standard IU mg^{-1})		Storage temperature (°C)	Present experiments (calculated from peak area)	1985 experiments (calculated from peak area)
CTC	(1000)	20	1011.5	1001.0
		37	943.8	938.0
OTC	(900)	20	880.9	873.9
		37	679.5	784.8[a]
TC	(982)	37	998.8	986.9
		56	990.3	968.2
DEM	(1000)	20	996.2	1014.0
		37	1016.5	1013.0
MET	(924)	37	928.0	926.7
		56	942.3	899.0
MIN	(863)	37	797.0	839.8[a]
		56	536.0	633.4[a]

[a] In these cases degradation has obviously occurred. For all other experiments the coefficients of variation between the mean of the two values are within 2%.

had degraded over the 3-year period, for example OTC and MIN, this was obvious from the peak area values.

The main advantage of a chemical assay is that results can be obtained rapidly. The retention times of the tetracycline samples on the column were short with a maximum run time of 17 min (flow-rate, 1.5 ml min^{-1}) being required in the case of MIN. Replicates of samples and standards could be run in a few hours. This compares favourably with the overnight incubation required with the microbiological assay. With the HPLC assay the degree of degradation can be calculated immediately by comparison of the integration values of the standard material and the test. Establishing degradation with the bioassay is more complicated. A degraded sample compared to the standard will give a potency ratio of less than 1 by bioassay, which will make the assay statistically unacceptable. To check the robustness of the assay, the procedure can then be repeated using an adjusted assumed potency value for the degraded sample. If this gives a potency ratio close to 1, it can be assumed that the decreased potency of the test sample is due to degradation and not to errors in the assay system. This was exemplified in the case of MIN. A further problem which may arise when using bioassay to measure degradation is that the degradation product(s) may also have antimicrobial properties and the results of the bioassay will not be a true representation of the antimicrobial potency of the original material. Degradation products are less likely to confuse the results of the HPLC analysis. The degradation products were not observed by HPLC analysis either because they might be absorbed on the column and not eluted or because breakdown may affect the UV absorbing properties of the resulting products. Degradation is thus most clearly observed through lower integration values for peak areas and peak heights and smaller peaks on a chromatogram (Fig. 3). Therefore the complex adjustments to the bioassay
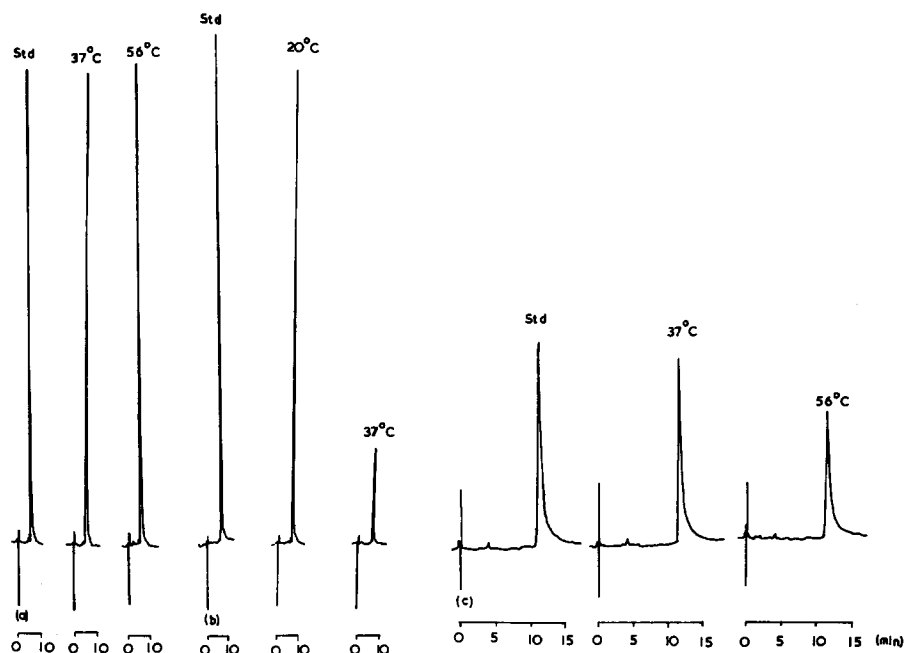
Fig. 3. Chromatograms of (a) TC showing no degradation, (b) OTC and (c) MIN both showing increased degradation at elevated temperatures. Std = standard stored at −20°C; wavelength = 280 nm; a.u.f.s. = 0.16.

procedure required for degraded compounds are not needed for HPLC assay, making the latter a more rapid and cost-effective method.

From Table I it can be seen that with the exception of OTC (stored at 37°C) and MIN, the tetracycline preparations show little significant degradation at elevated storage temperatures. The table shows however, that the agreement between potency values calculated by HPLC and bioassay for MIN is not as good as that for other preparations. MIN samples stored at 37°C and 56°C had a carbonised appearance and were brown and black respectively as compared with the yellow colour of the standard. The samples were prepared by dissolution in water followed by repeated sonication in order to ensure maximum solubilisation.

From the chromatograms shown in Fig. 3, the decrease in peak height with increase in storage temperature in the cases of OTC and MIN can be seen. The six replicate values for each sample were reproducible with coefficients of variation less than 4%, with the exception of CTC (coefficient of variation, C.V. = 7%). Since no degradation product of MIN was detected by HPLC, a spectrophotometric scan from 190 to 700 nm was carried out and showed no difference in absorption maxima (344, 277 and 247 nm) between the standard and both samples stored at 37°C and 56°C. Thus it seems that at least in the case of MIN, degradation did not lead to any compounds resolved in this HPLC system.

The column is maintained at 40°C during HPLC analysis to prevent the oxalate buffer from precipitating and to improve resolution of the peaks, although analyses can be performed at room temperature provided that the column is thoroughly washed

to prevent blockage with ammonium oxalate and thus reducing unnecessary wear on the pump seals.

The results reported here are in accordance with other comparative studies of HPLC and microbiological assays of tetracycline[6] and other antibiotics[7]. There are many examples in the literature of the use of HPLC for the assay of tetracyclines in animal tissue[6,8] and foodstuffs such as honey[9]. It has been reported[6] that the HPLC assay of CTC in pig tissues proved to be more reliable and offered greater sensitivity than three bioassay techniques. However it should be pointed out that the three microbiological assays: swab-on-premises, microbial inhibitor test and thin-layer chromatography–bioautography, reported by Korsund and MacNeil[6] are not of the same precision as the British Pharmacopoeia recommended bioassay[5] used here and would not therefore be expected to compare favourably with an HPLC assay.

CONCLUSION

The comparative study presented here shows that the chemical assay of members of the tetracycline group has several practical advantages over the bioassay and that the results of both methods are in good agreement (Fig. 2). The HPLC method can be easily adapted for use with several commercial columns by minor adjustments to running conditions such as flow-rate and temperature.

ACKNOWLEDGEMENT

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