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High-performance liquid chromatographic validated assay of doxorubicin in rat plasma and tissues

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Abstract

A specific and selective high-performance liquid chromatography (HPLC) technique that requires few manipulations, and is readily adaptable to analysis for a large series of samples, has been developed for the determination of the concentration of the anticancer drug doxorubicin (DXR) in rat serum and tissues. The biological samples were efficiently deproteinised and resolved from a reversed-phase nucleosil C_{18} column with fluorescence detection. The validation study of the proposed method was successfully carried out in an assay range of between 5 and 5000 ng/ml and was subsequently implemented in a pharmacokinetic study of DXR in Wistar rats that were treated by intravenous administration of the drug. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Doxorubicin (DXR) is an anthraquinone anticancer agent that is composed of an amino sugar (daunosamine) linked by an *O*-glycosidic bond to an aglycone (doxorubicinone). The drug has been used for more than 20 years in the treatment of patients with certain types of leukaemias, lymphomas, soft tissue sarcomas, solid tumours, etc. Unfortunately, the clinical use of DXR is limited by its toxicity, such as cumulative dose-related cardiotoxicity, myelosuppression and the development of drug resistance [1–3].

DXR is rapidly and broadly distributed in humans (V_d=20–30 l/kg) and is accumulated in irrigated tissues such as liver, lung, kidney, etc. and especially heart. Accordingly, from the pharmacokinetic point of view, its cardiotoxicity can be explained in terms of massive binding in the myocardium, justifying interest in studying the distribution of DXR in different organs and tissues, and especially in the heart [3–6]. Hence, as part of an investigation of cardiac toxicity caused by DXR was the need to find out whether there was a correlation between histological and echocardiographic changes of heart tissue and pharmacokinetic data.

Numerous high-performance liquid chromatographic (HPLC) methods for the analysis of DXR have been published. These methods are hampered by the involved and laborious solid- or liquid-phase extraction procedures required for sample clean-up [7–11]. Moreover, DXR is a very unstable drug in solution [12] and the samples should thus be ready

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for injection in the shortest time possible. From this point of view and in comparison with other assays for the bioanalysis of anthracyclins, the proposed method has the advantage that is a simple, rapid, specific and reliable analytical technique to determine DXR in biological samples by HPLC, both in plasma and in tissues such kidney, liver, lung, muscle and heart.

After developing and setting up the analytical technique, this was applied in the quantification of DXR in samples from rats, affording satisfactory results for pharmacokinetic studies.

2. Experimental

2.1. HPLC instrumentation and conditions

The chromatographic equipment comprised a Kontron pump (Model 420) coupled to a Shimadzu fluorescence detector (Model RF-10AXL). Data collection was accomplished with a Varian integrator (Model 4270).

Reverse-phase nucleosil C_{18} columns (25×0.4 cm) (Teknokroma, Barcelona, Spain) with a particle size of 10 μm were used. The mobile phase was composed of a mixture (65:35, v/v) of methanol and 0.01 *M* phosphate buffer, adjusted to pH 2.96±0.01 with 19 *M* potassium hydroxide in a Crison pH-meter (Model 2001). This mobile phase was prepared daily, filtered in a Supelco vacuum system (Model 5-8068) with a 0.45-μm nylon filter (Whatman, Maidstone, UK) and degassed in a P-Selecta ultrasound bath (Model M-515). The flow-rate during the assays was 2 ml/min, with an average working pressure of 2499±147 p.s.i. (1 p.s.i.= 6894.76 Pa), detection being accomplished at $\lambda_{ex}470/\lambda_{em}555$. The process was carried out at room temperature (20±5°C).

2.2. Chemicals and reagents

A lyophilised preparation for in situ reconstitution of DXR hydrochloride (Farmiblastina®) was supplied by Farmitalia (Pharmacia, Madrid, Spain). The mobile phase was prepared with HPLC–grade methanol (Fisher-Scientific, Leicestershire, UK) and 85% orthophosphoric acid from Panreac (Barcelona, Spain). The pH was adjusted with potassium hydroxide (Panreac). The deproteinising reagent was prepared using analytical $ZnSO_4$ (Panreac) and twice-destilled water.

2.3. Preparation of standards

After preparing a stock solution of 5000 ng/ml of DXR in blank human plasma, two solutions containing 1000 and 100 ng/ml DXR were obtained by successive dilutions. These three solutions served to obtain standard samples corresponding to the three concentration ranges studied (5–75, 50–600 and 500–5000 ng/ml) in the validation assay. Each concentration range contained five or six standard solutions (5–75: 5, 15, 30, 50 and 75 ng/ml; 50–600: 50, 100, 200, 400 and 600 ng/ml, and 500–5000: 500, 1000, 2000, 3000, 4000 and 5000 ng/ml), which were measured under conditions of sensitivity and gain established in the detector.

We could not use stock quality controls because of the labile nature of the drug. Therefore, three frozen samples that had been quantitated a day before were analyzed immediately and on the following day with calibration standards to determine new concentrations. We accepted values that were within ±10% of the known value.

2.4. Deproteinising procedure and sample preparation

Before the samples were injected into the chromatograph, protein denaturing and precipitation procedures were carried out. Sample treatment was as follows: 200 μl of a 50:50 (v/v) mixture of methanol –40% $ZnSO_4$ were added to 150 μl of sample (plasma or homogenised tissue preparation). After 1 min of vigorous vortex-mixing (Supermixer, Model 1291), the fluid was centrifuged using a Labofuge centrifuge (Model 6000) at 1500 *g* for 10 min. The supernatant thus obtained was injected directly into the chromatograph with a 200-μl fixed-volume loop.

Before being subjected to deproteinisation, the tissues were homogenised using a Scientific Inc. mechanical homogenizer (Model Pro 250) and diluted in 1/15 *M* phosphate buffer (pH 7.4±0.1). The volume of the buffer was determined as a function of

the density and characteristics of each tissue. The final concentrations of the tissues prior to their evaluation were 50 mg/ml for kidney and liver, 25 mg/ml for heart and lung, and 15 mg/ml for muscle.

2.5. Recovery, calibration and quantitation

Analytical and calibration standards were analysed in the same chromatographic run during validation to calculate absolute recoveries. The standards were calibrated using a linear fit program, with the following equations (response)$=mx+b$, where m=slope, b=intercept, x=concentration of DXR in ng/ml and (response) is the height or area peak. To solve for x=(response$-b)/m$, the program offered us the correlation coefficient and the determination coefficient. Moreover, the analysis of the coefficient of variation of the relative factor response (F.R.: quotient between height or area peak and theoretical concentration) and the study of the coefficient of variation (%C.V.) by one-way ANOVA served to confirm the linearity of the calibration curve for the three concentration ranges used, i.e. 5–75, 50–600 and 500–5000 ng/ml.

2.6. Selectivity and interferences

The selectivity of the method for measuring the sample specifically without interferences was determined by comparative study under different deproteinising conditions and percentages of methanol in the mobile phase. We compared the protein denaturing effect of acetonitrile, trichloroacetic acid (20%) and a mixture (50:50, v/v) of methanol–40% $ZnSO_4$. The deproteinising effect and the stability of the drug were suitable using methanol–40%$ZnSO_4$. We evaluated the percentage of methanol of the mobile phase carefully in order to reduce the interference from the plasma front.

2.7. Accuracy and precision

To set up the precision study, we analysed instrumental precision (variability in the response of the set-up to the same sample) and the precision of the method (variability in the response of the set-up to freshly prepared samples). The responses were evaluated in triplicate for the different concentration ranges and in the within- and between-day follow-up (three days).

Accuracy was evaluated by analysing absolute recovery percentages. Recoveries were calculated by comparing the area and height peak of the deproteinised plasma standard curve to that of the analytical standard curve of identical theoretical concentrations. We studied recovery data in a within- and between-day follow-up.

2.8. Stability of samples

The stability of DXR was tested in frozen biological samples and plasma standards during short-term storage, as well as in deproteinising solvent. Long-term stability has not been determined.

3. Results

3.1. Conditions and criteria of validation

The validation study was carried out in human plasma, and the methodology was applied subsequently to the determination of DXR in real rat plasma and tissues. We compared the recovery of 100 ml of 400 ng/ml DXR solution on phosphate buffer (pH 7.4±0.1) on 300 ml of blank human plasma, blank rat plasma and blank homogenised tissue preparation (heart, kidney, liver, lung, muscle) after 30 min of incubation at room temperature. The rat sample height and area peaks were between 95.6 and 97.3% of those corresponding to the human plasma, and the differences were not statistically significant at the 95% level according to Student's *t*-test. DXR appeared to be chemically stable in these fluids and also can be assumed to be fully released from proteins independent of the type of biological sample tested (rat serum, rat homogenised tissue preparation or human plasma). Therefore, after preparing a split standard curve in each of the biological fluids used, we confirmed that the height and area peaks were not statistically significant at the 95% level according to Student's *t*-test. The main reason for using human plasma instead of rat serum or tissues for the validating the analytical technique is the easier availability of large volumes of human plasma compared to rat plasma or tissue.

Since the detector response relationship is linear throughout the concentration range we were interested in and a possible internal standard (daunorubicin) did not improve reproducibility, we employed an external standard for determining DXR in the samples, from which, the unknown concentrations were calculated as directly proportional.

3.2. Selectivity and interferences

Irrespective of the biological fluids used, we observed good resolution of the peaks and the absence of interferences permitted easy and clear identification of the drug. Fig. 1 shows the chromatographic peaks corresponding to 5, 50 and 5000 ng/ml of DXR in human plasma and reflects the specificity and selectivity of the analytical technique.

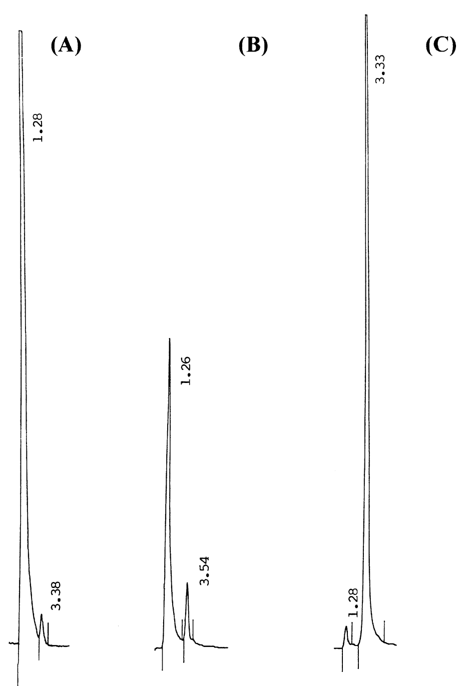

Fig. 1. Chromatographic peaks of DXR in plasma (retention time, 3.38±0.22 min): (A) 5 ng/ml, (B) 50 ng/ml and (C) 5000 ng/ml. Chromatographic conditions: 65% MeOH– 35% 0.01 *M* H_3PO_4; flow-rate, 2 ml/min; temperature, 20±5°C. Fluorescence detection: wavelength, $\lambda_{ex}470/\lambda_{em}555$; sensitivity (S) and gain (G) parameters, 5 ng/ml (S1G2), 50 ng/ml (S1G1), 5000 ng/ml (S2G2).

DXR elutes rapidly after the plasma front, with a retention time of 3.38±0.22 min.

Three syringe washes of methanol and distilled water after each sample injection, regular maintenance of the HPLC column and fluorescence detector prevented interferences in the analysis of the increased number of samples.

3.3. Limits of detection and quantification

The limit of detection for this assay was approximately 1 ng/ml, irrespective of the type of biological sample tested, but the precision of the concentration determination was poor at this low concentration, due to background noise interference. Analyte samples with less than 5 ng/ml and more than 1 ng/ml could be detected but not quantified with precision and accuracy. Following the analysis of ten blanks, a limit of quantification of 5 ng/ml was determined with a confidence level of 95%, with the mean value and standard deviation of the response being 5.36±0.25 ng/ml.

3.4. Linearity

Over the range of 5–5000 ng/ml, a linear fit was used with satisfactory results. The data were fitted to a line by the equation $y=mx+b$. Table 1 expresses the parameters of the equations obtained in the regression study for each concentration in a between-day (three days) follow-up.

Analysis of the F.R. served to check the linearity of calibrations. Table 2 shows the %C.V. of the F.R. in a between-day study. As can be seen, the %C.V. in the study, by height, is lower than that obtained by area, in this case, being less than 6%.

The coefficient of variation (%C.V.) performed using one-way ANOVA confirmed the excellent linearity of the calibration curves in a within- and between-day study. The study by height ranged from 4 to 7%, whereas the study by area ranged from 3 to 11%. It is probable that the short retention time, which afforded good peak isolation and resolution, justifies the better data obtained from the height peak study.

Table 1
Regression linearity fit by the equation ($y=mx+b$), where y=height or area peak, m=slope, b=intercept, x=drug concentration (ng/ml), r=correlation coefficient and r^2=determination coefficient

Range of concentrations (ng/ml)	Height				Area			
	m	b	r	r^2	m	b	r	r^2
5–75	6.10E+02 ±6.27	−3.49E+02 ±2.71	0.999	0.999	2.08E+04 ±6.35E+02	−3.77E+04 ±2.74E+04	0.997	0.998
50–600	1.54E+02 ±2.34	−4.29E+02 ±7.92+02	0.999	0.997	4.98E+03 ±6.06E+01	−3.12E+04 ±2.05E+04	0.999	0.998
500–5000	2.24E+01± 5.31E−01	−1.56E+03 ±1.61E+03	0.996	0.991	7.59E+02 ±8.36	−1.05E+05 ±2.54E+04	0.999	0.998

Table 2
Study of the relative factor response (F.R.) in a between-day study (x, mean value; SD, standard deviation; %C.V., coefficient of variation)

	5–75 ng/ml			50–600 ng/ml			500–5000 ng/ml		
	x	SD	%C.V.	x	SD	%C.V.	x	SD	%C.V.
Heights	589.42	24.03	4.08	155.58	9.17	5.89	21.56	1.12	5.20
Areas	18674.40	2218.37	11.88	4713.78	361.71	7.67	686.03	65.96	9.42

3.5. Accuracy and precision

Table 3 shows the study of the instrument's precision and the precision of the method. The results for the within-day study point to the good precision of both the set-up and the methodology used, the %C.V. by height not being greater than 6% in both cases.

The between-day (three days) study of the instrument's precision showed a high variability of %C.V., so that our experimental results confirmed the labile nature of this drug. It was therefore expedient to

Table 3
Within-day and between-day variability. Mean coefficient of variation (%) and standard deviation of the instrument's precision and the precision of the method

			5–75 ng/ml	50–600 ng/ml	500–5000 ng/ml
Within-day	Instrument's precision (% C.V.)	Height	3.84±0.87	3.28±1.19	4.37±2.47
		Area	6.25±3.52	5.27±4.98	6.15±1.79
	Precision of the method (% C.V.)	Height	5.64±3.05	3.51±1.18	5.73±1.59
		Area	8.79±7.69	6.48±3.00	5.08±1.24
Between-day	Instrument's precision (% C.V.)	Height	14.77±6.34	25.62±6.04	9.55±2.52
		Area	10.58±5.60	15.24±4.90	3.63±1.42
	Precision of the method (% C.V.)	Height	3.16±2.51	7.09±5.62	4.68±1.61
		Area	8.05±4.02	5.44±3.75	3.75±1.94

Table 4
Statistics generated during validation for within-day ($n=3$) accuracy study by height and area (x, mean value; %C.V., coefficient of variation; %R.E., relative error; %M.R., mean recovery)

Concentration of sample (ng/ml)	Height				Area			
	x	%C.V.	%R.E.	%M.R.	x	%C.V.	%R.E.	%M.R.
5	4.93	5.13	−1.33	98.69	4.47	3.81	−10.67	89.33
15	15.27	10.17	+1.82	10.18E+01	15.60	21.84	+3.98	10.40E+01
30	29.65	3.30	−1.16	98.85	30.21	6.35	+0.69	10.07E+01
50	50.15	4.72	+0.13	10.03E+01	49.69	5.45	−0.62	99.38
75	74.99	3.23	−0.02	99.98	75.04	5.30	+0.05	10.01E+01
50	50.79	9.27	+1.59	10.16E+01	54.73	2.20	+9.45	10.95E+01
100	10.10E+01	4.13	+1.03	10.10E+01	10.45E+01	3.36	+4.48	10.45E+01
200	19.70E+01	2.41	−1.52	98.47	18.78E+01	5.30	−6.09	93.90
400	40.14E+01	3.78	+0.34	10.03E+01	40.02E+01	9.62	+0.04	10.01E+01
600	59.99E+01	1.83	−0.02	99.98	60.28E+01	8.82	+0.47	10.05E+01
500	55.11E+01	4.70	+10.23	11.02E+01	54.21E+01	3.39	+8.42	10.84E+01
1000	10.17E+02	6.23	+1.68	10.17E+01	99.45E+01	4.28	−0.55	99.45
2000	19.67E+02	6.74	−1.65	98.70	20.08E+02	5.51	+0.38	10.04E+01
3000	29.08E+02	6.23	+3.07	96.93	29.12E+02	6.29	−2.92	97.08
4000	39.86E+02	4.32	−0.35	99.65	39.85E+02	4.13	−0.37	99.63
5000	50.71E+02	3.11	+1.42	10.14E+01	50.58E+02	3.08	+1.17	10.12E+01

condition the samples immediately before their determination. Based on this reasoning, we could explain the good precision of the method obtained in a between-day study.

Accuracy was evaluated using ANOVA to yield the absolute recovery percentages of the amount of DXR present in the sample and the corresponding %C.V. Within- and between-day statistics including

Table 5
Statistics generated during validation for between-day ($n=3$) accuracy study by height and area (x, mean value; %C.V., coefficient of variation; %R.E., relative error; %M.R., mean recovery)

Concentration of sample (ng/ml)	Height				Area			
	x	%C.V.	%R.E.	%M.R.	x	%C.V.	%R.E.	%M.R.
5	5.08	2.65	+1.50	1051E+01	5.51	4.11	+10.20	11.52E+01
15	14.79	1.49	−1.42	98.55	14.21	8.41	−5.27	94.74
30	29.32	4.44	−2.28	97.72	28.77	2.71	−4.11	95.90
50	51.04	1.98	+2.07	10.21E+01	51.98	11.00	+3.97	10.40E+01
75	74.61	1.26	−0.52	99.48	74.28	4.56	−0.96	99.04
50	46.71	13.10	−6.57	93.42	49.02	9.50	−1.97	98.03
100	96.48	5.83	−3.52	96.48	98.43	6.46	−1.57	98.43
200	20.32E+01	3.45	+1.62	10.16E+01	20.01E+01	5.10	+0.03	10.00E+01
400	41.14E+01	3.89	+2.84	10.28E+01	40.65E+01	1.12	+1.63	10.16E+01
600	59.22E+01	2.52	−1.30	98.70	59.60E+01	3.00	−0.67	99.33
500	50.90E+01	2.29	+1.81	10.18E+01	51.84E+01	4.44	+3.68	10.11E+01
1000	98.90E+01	3.92	−1.10	98.90	97.39E+01	2.59	−2.21	97.39
2000	19.81E+02	3.95	−0.94	99.06	19.93E+02	4.56	−0.34	99.66
3000	30.28E+02	4.32	+0.92	10.09E+01	30.23E+02	5.00	+0.75	10.09E+01
4000	40.05E+02	5.61	+0.11	10.01E+01	39.97E+02	1.95	−0.08	99.46
5000	49.89E+02	7.19	−0.23	99.77	49.95E+02	1.18	−0.10	99.90

mean value, the %C.V. of the mean, the relative error (%) and the mean recovery (%) (see Tables 4 and 5). The recovery percentages thus obtained, which were close to 100%, confirm the accuracy of the technique and hence the excellent correlation between the theoretical and experimental values.

At the same time, the variation in the %C.V. with the theoretical concentration was studied. This pointed to an exponential relationship for the 5–75 and 50–600 ng/ml ranges, expressed as:

5–75 ng/ml;	By height	$\%\text{C.V.}=(16.43\pm9.81)\cdot C^{-0.57\pm0.26}$
	By area	$\%\text{C.V.}=(16.92\pm10.43)\cdot C^{-0.24\pm0.21}$
50–600 ng/ml;	By height	$\%\text{C.V.}=(48.55\text{E}+01\pm37.38\text{E}+01)\cdot C^{-0.87\pm0.18}$
	By area	$\%\text{C.V.}\ (14.26\text{E}+01\pm81.51)\cdot C^{-0.66\pm0.13}$

By contrast, no such relationship was observed for the 500–5000 ng/ml range. This situation could be explained in terms of the different behaviours of the detector when measuring high concentrations.

3.6. Stability of samples during deproteinising and analysis

The drug substance is stable for at least 15 days in frozen biological samples (−20°C) and for 1 h in the deproteinising solvent. Our between-day (three days) precision of the instrument results confirmed the labile nature of the drug. The heat- and light-sensitivity of the active ingredient [12] means that its stability cannot be guaranteed following long-term storage.

3.7. Proof of applicability

After the validation study, we applied the methodology to the determination of DXR in real plasma and tissue samples obtained from Wistar rats (mean weight, 240±10 g) treated intravenously with DXR (7.5 mg/kg). Samples of plasma, heart, liver, kidney, lung and muscle were collected. Prior conditioning of the tissue samples (homogenisation and dilution)

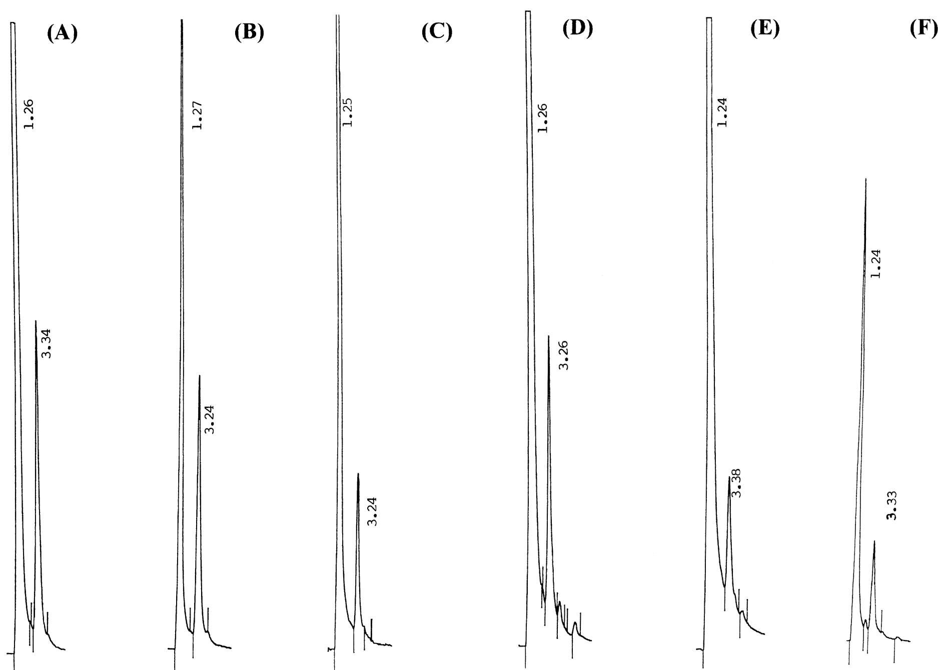

Fig. 2. Chromatographic peaks corresponding to different rat tissues (retention time, 3.38±0.22 min). Quantification of the DXR that had accumulated at 2 h after administration (DXR i.v., 7.5 mg/kg). (A) Heart, 134 82.00 ng/g tissue; (B) Lung, 111 79.20 ng/g tissue; (C) muscle, 2550.67 ng/g tissue; (D) liver, 5970.60 ng/g tissue; (E) kidney, 1967.20 ng/g tissue and (F) plasma, 108.22 ng/ml. Chromatographic conditions and sample preparation are as given in Section 2.

was carried out as a function of their individual characteristics.

Detection of DXR in rat plasma and tissues proved to be as simple and specific as for human plasma. DXR eluted after the solvent front, with very few variations in the retention time. The chromatographic peaks were well resolved and the specificity and selectivity of DXR isolation were excellent. In the chromatograms of liver and kidney samples, peaks other than that of DXR were also obtained; it is possible that these correspond to the appearance of metabolites such as doxorubicinol [7,9,11]. As seen in Fig. 2, the chromatograms obtained in the analysis of the different tissues confirm that the DXR accumulated in problem samples can be quantified.

Previously proofs and the continued use of this methodology helped us to choose the standard curve to employ for quantification the unknown samples. The data generated from preparing rat samples by this method was used to calculate pharmacokinetic parameters to support a comparative tissue distribution study of DXR.

4. Conclusions

A method for the quantification of DXR in rat samples has been developed and validated. The advantage of this assay is its simplicity. It requires a single solvent deproteinising step. Our results show the excellent linearity, precision and accuracy of the analytical technique. The concentration values obtained were then used to calculate pharmacokinetic data to support the DXR distribution and binding to heart.

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