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HPLC methods for the determination of bound and free doxorubicin, and of bound and free galactosamine, in methacrylamide polymer–drug conjugates

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Abstract

Quantitative acid hydrolysis followed by HPLC separation has been established as an analytical procedure for the determination of polymer-bound doxorubicin (an anti-cancer drug) and D-galactosamine (a liver-targetting moiety) in the polymer–drug conjugates FCE 28068 and FCE 28069. Optimal conditions of hydrolysis were determined in both cases: 1 N HCl, 50°C, 1.5 h for doxorubicin, and 6 N HCl, 60°C, 5 h for galactosamine. Appropriate HPLC quantitation of galactosamine required pre-treatment with sodium borohydride and pre-column derivatization with *o*-phthalalaldehyde and *β*-mercaptoethanol. Independent determination of free doxorubicin and glactosamine in untreated polymer samples was also achieved with the same HPLC method up to detection limits of 0.01% and 0.02% respectively. The methods were validated for linearity, precision and repeatability. Validation for accuracy before and after acid hydrolysis was achieved by testing hydrolysis on model compounds and by assessing recovery in polymer solutions spiked with free doxorubicin or galactosamine.

Keywords: Acid hydrolysis; Doxorubicin; D-Galactosamine; Liver targetting; Polymer–drug conjugates

1. Introduction

Polymer–drug conjugates are at the forefront of cancer chemotherapy and are being intensively investigated for a series of potential or established pharmacological benefits [1]: reduced toxicity, improved availability at tumour site, and selective organ targetting by means of carrier molecules covalently attached to the polymer. FCE 28068 and FCE 28069 (see Fig. 1) are methacrylamide copolymers which both contain the anti-cancer drug doxorubicin attached via a tetrapeptidyl spacer that is specifically designed for intracellular cleavage [2]. Furthermore, in the case of FCE 28069, molecules of D-galactosamine are bound via an amide linkage to some of the peptide chains in order to ensure liver targetting of the polymer [3].

Use of a non-selective method such as UV or visible light absorption for the quantitation of total doxorubicin content of polymer batches is possible but not advisable for two reasons.

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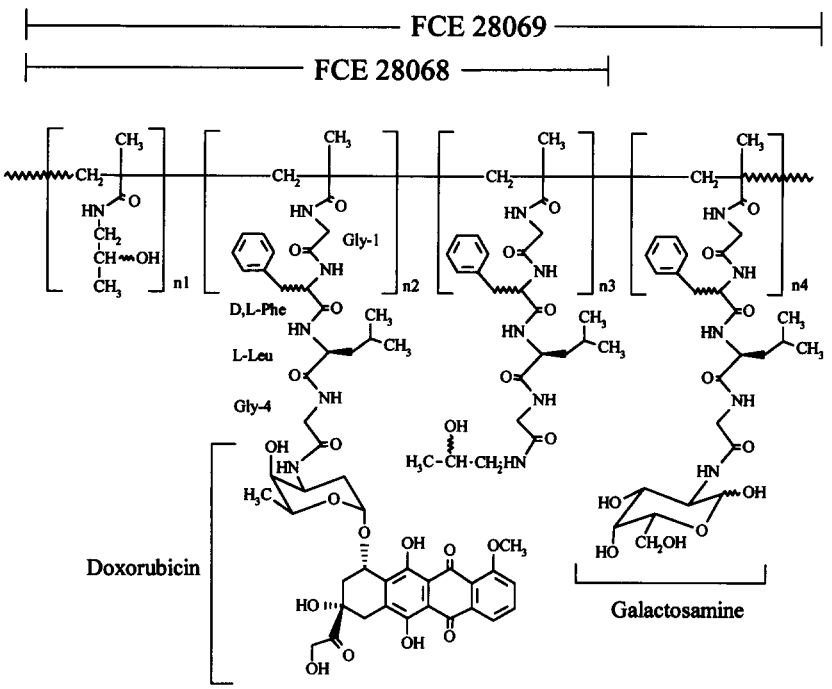

Fig. 1. Structures of FCE 28068 and FCE 28069.

Firstly, the extinction coefficient of free and polymer-bound drug may be different, and thus calibration with a free drug standard would lead to inaccurate results. Secondly, degradation products with similar light absorption properties would be incorrectly accounted for as active drug. Enzymatic hydrolysis of these polymer conjugates releases the free drug [4], but standardisation of this reaction up to a quantitative assay level proved very difficult, if possible at all. Therefore, analytical characterisation of these compounds in terms of total drug (or carrier) content had to rely on chemical cleavage of the bond between polymer and drug (or galactosamine) and subsequent quantitation of this latter moiety with a selective HPLC method. Validation of both assays for accuracy was not straightforward, in the absence of an independent determination of the total drug or galactosamine content in the polymer conjugates. The strategy adopted in this work was based on the following three steps: (1) quantitation of hydrolysis yield for a model compound with well characterised structure under optimised conditions; (2) comparison of the release kinetics obtained with polymers and model compounds; (3) determination of recovery in polymer solutions spiked with free doxorubicin or galactosamine. The first two steps attempted to demonstrate complete release of polymer-bound molecules, the third verified the stability and recoverability of the tested molecule under the conditions of chemical hydrolysis.

Complete development of the two analytical methods required consideration of the following aspects. In the case of the doxorubicin assay, the chemical entity which is released is not the drug but the corresponding aglycone, adriamycinone, as the labile glycosidic bond breaks first during hydrolysis [5]. Therefore, the total drug content of the polymer conjugates was assessed after calibration with an adriamycinone standard of known purity and by taking into account the molecular weight difference between the two chemical species. In the case of the galactosamine assay, acceptable HPLC behaviour was only obtained after reduction of the aminosugar to the corresponding alditol [6] and with pre-column derivatization using *o*-phthalaldehyde and *β*-mercaptoethanol [7].

Determination of free drug and free galactosamine in the untreated polymers was necessary for appropriate evaluation of these contaminants and for correct accounting of bound species (as difference between total and free forms) after the acid hydrolysis assay. To this purpose the same HPLC methods were here applied to polymer solutions before hydrolysis, and a high polymer concentration was used in order to maximise sensitivity.

2. Experimental

2.1. Materials

All samples of polymer–drug conjugates used in this study were provided by Pharmaceutical Research & Development of Pharmacia (Nerviano, Italy). Doxorubicin hydrochloride and adriamycinone were working standards with known purity. D-Galactosamine and *N*-acetyl-D-galactosamine were purchased from Sigma. 6 N HCl was obtained from Pierce, sodium borohydride from Fluka and *o*-phthalaldehyde (OPA) from Aldrich. All other solvents and chemicals were from Carlo Erba.

2.2. Instrumentation and HPLC separation

The liquid chromatograph was an HP1090 M provided with diode array detection and autosampler. For the assay of doxorubicin (free and total) the column was a Vydac C18 (250 mm × 4.6 mm) and separation was obtained with the following linear gradient of water (A)–acetonitrile (B), both containing 0.1% (v/v) CF_3COOH: from 25% B to 30% B in 10 min, and then up to 80% B in 5 min, elution was maintained isocratic for the next 15 min. For the galactosamine method the column was a Novapak C18 (150 mm × 3.9 mm, 4μ) and elution included an isocratic phase (5 min with 0.025 M KH_2PO_4, pH 6–acetonitrile: 95/5) followed by a linear gradient up to a phosphate–acetonitrile ratio of 40:60 in 25 min. The reactive mixture for pre-column derivatization of galactosamine (OPA) was prepared as follows: 27 mg of *o*-phthalaldehyde in 0.5 ml ethanol was dissolved with 5 ml of 0.4 M borate buffer, pH 9.4. After addition of 20 μl of β-mercaptoethanol, the solution was stored in the dark for one night and used within 1 week.

2.3. Sample preparation: doxorubicin

Polymer samples were dissolved in water at a concentration of about 5 mg ml^{-1} and a portion of this solution was used for the determination of free drug. Acid hydrolysis was carried out with 0.5 ml of this solution after mixing with an equal amount of 2 N HCl in a micro-reaction vessel (Supelco) heated at 50°C for 1.5 h. After this time the solution was cooled at room temperature and analysed within 2 h.

2.4. Sample preparation: galactosamine

An aliquot (0.5 ml) of FCE 28069 solution in water (about 10 mg ml^{-1}) was transferred into a screw cap tube (PBI, Bavis-type) and treated with 0.2 ml of a freshly made 1 M sodium borohydride solution in water. After 90 min at room temperature, the excess borohydride was eliminated with addition of 17 μl acetic acid in a fumehood (H_2 is released, tube uncapped!). After 30 min at room temperature the solvent was evaporated in a Savant centrifuge. For the determination of free galactosamine, the residue was resuspended in 1 ml of derivatization buffer (0.4 M borate, pH 9.4) and submitted to HPLC. Pre-column derivatization was accomplished with the HP injector program. Solutions of standard were treated similarly. For the determination of total galactosamine an aliquot of the FCE 28069 solution in water was treated with 0.2 ml of 1 M $NaBH_4$ and 0.3 ml water. After 90 min at room temperature, the excess borohydride was eliminated with addition of 6 N HCl (34 μl) and the solution evaporated to dryness. The residue was redissolved in 6 N HCl (1 ml) and the solution was saturated with argon before hydrolysis (60°C, 5 h, capped tube). Solvent removal in the Savant centrifuge and resuspension in derivatization buffer (1 ml), followed by filtration with a Millex HV13, preceded HPLC.

3. Results

3.1. Doxorubicin

Determination of contaminant-free doxorubicin is accomplished by HPLC of the aqueous solution of the polymers before hydrolysis. Separation of all relevant species (doxorubicin, adriamycinone, polymer conjugate) is shown in Fig. 2 for the case of FCE 28068. A high polymer concentration (5 mg ml^{-1}) is used for high sensitivity: under these conditions the limit of detection and limit of quantitation are 0.01% and 0.02% respectively, as assessed according to the criteria of $3\sigma/b$ and $5\sigma/b$ (standard deviation of data/slope of calibration line in the concentration range 1–10 μg ml^{-1}). The assay was validated by determining linearity with solutions of doxorubicin hydrochloride ($r^2 = 0.99987$ with eight solutions in the concentration range 1–50 μg ml^{-1}). Determination of recovery is shown in Fig. 3A, where results of free doxorubicin assay are plotted as a function of the known amount of added drug in spiked samples of FCE 28068. The average recovery (98.7%) is obtained from the slope of the regression line ($r^2 = 0.9993$), whereas the intercept quantifies the amount of free doxorubicin in that batch of FCE 28068 (0.05%). Precision of the assay is assessed with repeated determinations on solutions of doxorubicin hydrochloride (RSD of seven determinations with a 5.45 μg ml^{-1} solution = 0.75%) and with repeated tests of free doxorubicin content on the same batch of FCE 28068 (see Table 1).

Mild acid hydrolysis of doxorubicin (Fig. 4) selectively cleaves the glycosidic bond between the aglycone (adriamycinone) and the sugar (daunosamine). The much more stable amide bond between the aminosugar and the peptidyl chain requires much higher acid concentration and temperature for effective hydrolysis, and thus quantitation of bound doxorubicin after chemical hydrolysis is made on the basis of released adriamycinone. Optimisation of hydrolytic conditions was carried out with doxorubicin hydrochloride and 1 N HCl, 50°C, 1.5 h was found to be the best compromise between maximal conversion (> 99%) and stability of the formed product. Fig. 5 shows the conversion yield as a function of time during acid hydrolysis of doxorubicin at 40, 50 and 60°C, and for comparison that of FCE 28068 at 50°C. In the model study, maximum yield is not observed within 3 h at 40 °C, whereas this is rapidly obtained at the higher temperatures where a plateau of around 100% conversion is reached between 1 and 2 h of hydrolysis, with some evi-

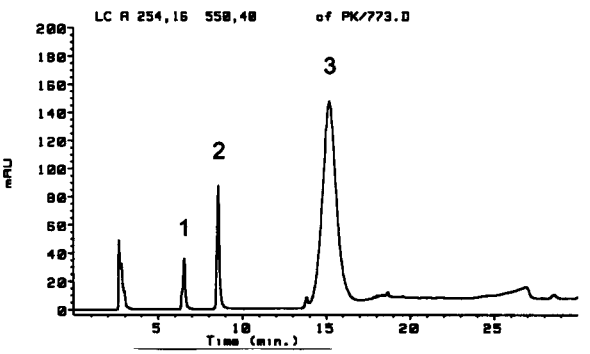

Fig. 2. HPLC separation of doxorubicin (1), adriamycinone (2) and FCE 28068 (3) on a Vydac C18 column. See Section 2 for details.

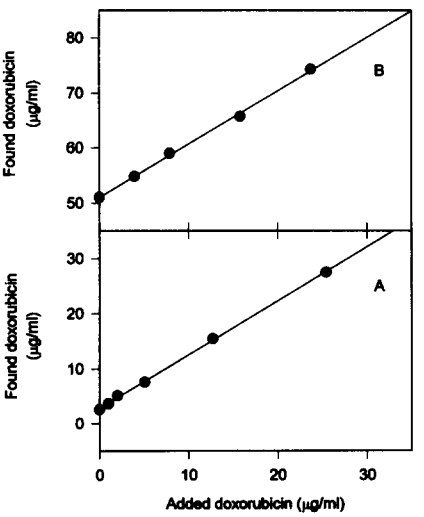

Fig. 3. Evaluation of recovery in the assay for free (A) and total (B) doxorubicin in FCE 28068. Samples with a constant polymer concentration were spiked with known amounts of doxorubicin hydrochloride and submitted to the two analytical procedures. The results are reported as plots found vs. known added concentrations of doxorubicin and the recovery (97.7% and 96.9% for free and total doxorubicin respectively) is obtained from the slope of the best-fitting straight lines. The intercepts correspond to the amount of free (0.05%) or total (7.9%) doxorubicin originally present in the polymer sample.

Table 1
Day-to-day reproducibility of total doxorubicin determination in FCE 28068 (two different batches were used for the total and free assay)

Day	Free doxorubicin % (w/w)	Total doxorubicin % (w/w)
1	0.024	8.61
2	0.026	8.46
3	0.020	8.30
4	0.020	8.30
5	Not assayed	8.34
6	Not assayed	8.65
Average	0.023	8.43
SD	0.003	0.17
RSD (%)	13.0	2.0

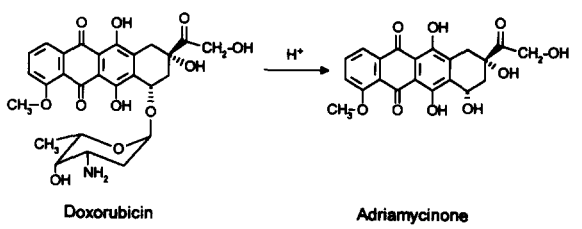

Fig. 4. Acid hydrolysis of doxorubicin leading to formation of the aglycone, adriamycinone.

dence of decreasing yield (probably due to degradation of the formed adriamycinone) later on. Hydrolysis of FCE 28068 at 50°C also leads to a plateau of maximum yield between 1 and 2 h. Results obtained with FCE 28069 are very similar and are not reported. The thus-formed adriamycinone is completely solubilized with addition of DMSO (three volumes) and this solution is stable at room temperature for 2–3 h and suitable for injection onto the HPLC column. Calibration is accomplished with adriamycinone in DMSO/water (3:1) between 5 and 50 μg ml^{-1}, where good linearity is observed ($r^2 = 0.99997$, five data points). The amount of total doxorubicin free base in the sample is obtained after multiplication with a conversion factor (1.311) which takes into account the different molecular weights of the two species.

As no better method was available for the quantitation of polymer-bound doxorubicin, accuracy of the assay was determined by evaluating the recovery with artificially made mixtures of polymer conjugate and free drug. Results are illustrated in Fig. 3B, where the amount of total doxorubicin found is plotted vs. the known added amount. The intercept of the line corresponds to the total doxorubicin content of the pure polymer preparation (7.9%), and the slope of the straight line ($r^2 = 0.9997$) indicates an average recovery of 96.9%. Precision of the assay is illustrated by data on reproducibility (see Table 1): repeated analyses of the same FCE 28068 batch on six different days lead to a mean total doxorubicin content of 8.4% (w/w), with a relative standard deviation of 2.0%.

3.2. Galactosamine

Derivatization of galactosamine with OPA and β-mercaptoethanol lead to poor HPLC behaviour unless pre-treatment with sodium borohydride was introduced to eliminate anomeric equilibrium and interconversion between furanose, pyranose and open forms [6]. In the case of total galactosamine determination, the best results were obtained when the reduction step was the first

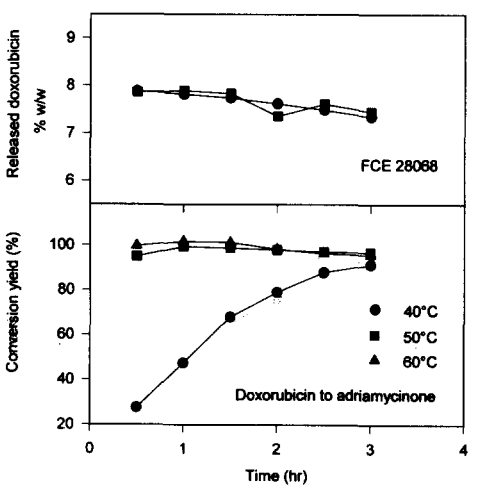

Fig. 5. Comparative kinetics of acid hydrolysis for doxorubicin in 1 N HCl at three different temperatures and for FCE 28068 in 1 N HCl, 50°C. Results (reaction yield for doxorubicin, released drug for FCE 28068) are the average of two independent sets of experiments for the doxorubicin kinetics, whereas two separate experiments are reported in the case of the polymer–drug conjugate.

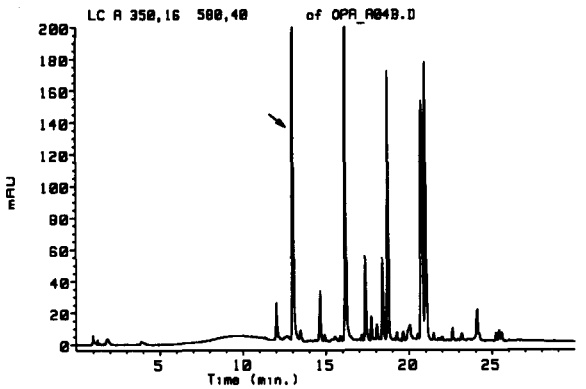

Fig. 6. HPLC separation of reduced galactosamine (peak marked with an arrow) from other hydrolysis products of FCE 28069 after derivatization with OPA. Identification of galactosamine peak was confirmed by coelution in spiked samples.

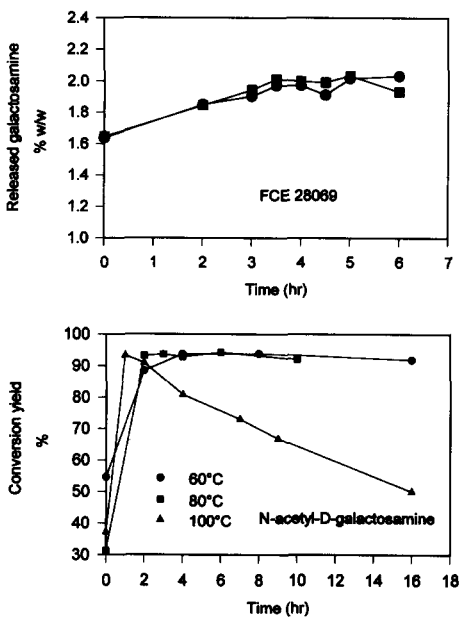

Fig. 7. Galactosamine yield in acid hydrolysis of the model compound *N*-acetyl-galactosamine (60, 80, 100°C and 6 N HCl) is compared with yield during hydrolysis of FCE 28069 (60°C, 6 N HCl).

treatment, preceding acid hydrolysis. With the opposite sequence (first hydrolysis and then reduction) formation of a precipitate and poor recovery were obtained. An example of chromatographic results for the acid hydrolysate of FCE 28069 is shown in Fig. 6. As assessed with data for a galactosamine standard, the chromatographic assay is linear in the range 8–170 μg ml^{-1} ($r^2 = 0.9996$, six data points) and reproducible with a precision of 1.7% (RSD of seven determinations with a 42 μg ml^{-1} solution of galactosamine). Limits of detection (LOD) and quantitation (LOQ) are established as above

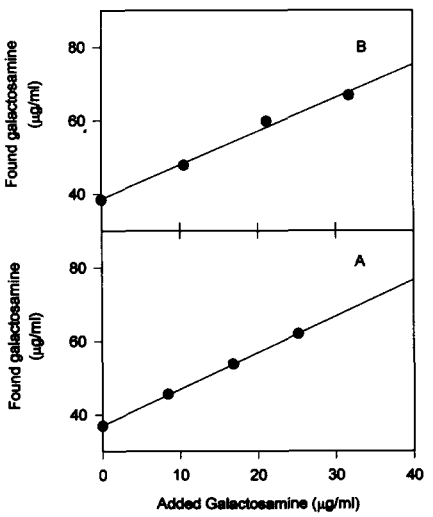

Fig. 8. Evaluation of recovery in the assays for free (A) and total (B) galactosamine content of FCE 28069. Samples with a constant concentration of this polymer were spiked with increasing amounts of galactosamine and submitted to the two analytical procedures (different batches of FCE 28069 were used in the two cases). The results are reported as a plot of found vs. known added concentrations of galactosamine. The interpretation of the slope and intercept of the best-fitting straight lines is identical to that described in Fig. 3 and values of recovery are 99.3% and 91.7% for the free and total galactosamine assay respectively.

Table 2
Day-to-day reproducibility of free and total galactosamine determination in FCE 28069 (two different batches were used for the free and total assay)

Day	Free galactosamine % (w/w)	Total galactosamine % (w/w)
1	0.69	1.88
2	0.65	1.87
3	0.69	1.84
4	0.65	1.75
Average	0.67	1.84
RSD (%)	3.0	3.3

(LOD = 1 μg ml^{-1} LOQ = 2 μg ml^{-1}). These correspond to 0.02% and 0.04% for a typical FCE 28069 solution used in free galactosamine determination (5 mg ml^{-1}).

Free galactosamine is released from FCE 28069 only after heating under strongly acidic conditions (6 N HCl), as a consequence of the good stability of the amide bond which connects it to the polymer side chains. Optimisation of hydrolytic conditions was carried out with a model compound, *N*-acetyl-D-galactosamine, whose kinetics of hydrolysis in 6 N HCl were investigated at three different temperatures (Fig. 7). At the highest temperature, conversion yield rapidly decreases with increasing reaction time, most likely as a consequence of chemical degradation. Results at 80°C or 60°C led to comparable plateau values, with some indication of improved stability at the lowest temperature, which was thus selected as the most suitable. The stability of galactosamine in 6 N HCl at 60°C was independently investigated and found to be satisfactory over a period of 10 h (data not shown). Maximum conversion yield (never exceeding 94%) probably accounts in part for the relative purity of the two commercial samples (manufacturer's claims: *N*-acetylgalactosamine, 98%; galactosamine, 99%). Nevertheless, this value sets a limit on the accuracy of the method. The release of galactosamine from a sample of FCE 28069 heated at 60°C in 6 N HCl is also shown in Fig. 7. Consistent with data on the model compound, a plateau is reached after about 4 h.

As in the case of doxorubicin analysis, recovery was tested with polymer solutions spiked with known amounts of galactosamine. For both free and total galactosamine assay, the results are shown in Fig. 8 as linear plots of found galactosamine vs. known added amount: the slopes of the straight lines ($r^2 = 0.9998$ and 0.991 for free and total galactosamine respectively) indicate average recoveries of 99.3% and 91.7%; the intercepts correspond to the galactosamine originally present in the two polymer preparations (1.84% total galactosamine in batch 0109, 0.67% free galactosamine in batch 0015). Both recovery and correlation coefficient are much poorer in the determination of total galactosamine than in the less elaborate assay of free galactosamine, most likely as a consequence of the hydrolysis step. However, precision of the two assays is comparable (RSD $\approx$ 3%), as shown by data of day-to-day repeatability which are reported in Table 2.

4. Conclusions

The results presented above demonstrate that analytical characterisation of polymer–drug conjugates by HPLC is feasible up to the level of commonly accepted validation standards for new drugs under investigation. Optimisation of hydrolytic conditions and validation of the accuracy by testing on low molecular weight model compounds proved crucial to a correct method development. High sensitivity detection of free drug and galactosamine is necessary both for evaluation of these contaminants and for proper accounting of the bound forms as differences between total and free amounts. In the present method, the actual precision of free drug or carrier determination is about five-fold higher than that of the total value, and thus the error for the bound forms (as the difference between total and free forms) is practically identical to that of the assay for the total. Finally, this approach, which includes a combination of two assays (independently determined free and total drug or free and total galactosamine), appears the only adequate tool for the monitoring of stability studies, where both the release as well as the degradation of polymer-bound species can occur.

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