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Short Communication

Quantitation of the dexrazoxane hydrolysis product ADR-925 by fluorescence detection of its terbium(III) complex after high-performance liquid chromatographic separation

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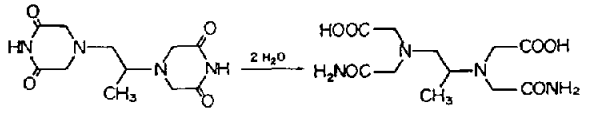
Abstract

An HPLC fluorescence detection method was developed to quantitate the complexing agent ADR-925 (II). Compound II is the metal-ion-binding rings-opened hydrolysis product of the doxorubicin cardioprotective drug dexrazoxane (I). II formed a strong complex with the fluorescent metal-ion terbium(III) and this complex could be chromatographed by HPLC and detected by its fluorescence, with excitation and emission wavelengths of 200 and 544 nm, respectively. The terbium(III)–II complex was separated isocratically on a C_{18} reversed-phase column with an eluent consisting of 50% methanol and 50% 4 m*M* aqueous solution of the ion-pairing reagent 1-heptanesulfonate. The lower limit of detection of II, quantitated as its fluorescent terbium(III) complex, was estimated to be 25 pmol, which was some twenty times lower than with UV-Vis absorbance detection. The fluorescent detection method was used to follow the hydrolysis of I to II in buffer and in blood plasma.

1. Introduction

Dexrazoxane (ICRF-187[1], I) has been shown in clinical trials [1] to be very effective in preventing doxorubicin-induced cardiotoxicity. This toxicity is thought to be due to an iron-dependent oxidative stress on the cardiac muscle [2,3]. I is the (+)-(*S*)-enantiomer of razoxane (ICRF-159), which upon full hydrolysis yields II (Fig. 1). II has a structure similar to EDTA and is, likewise, a strong metal-ion chelating agent [4]. I likely acts by diffusing into the cell [5], hydrolyzing to its presumably active rings-opened metal-ion-binding form and chelating free iron or iron bound to the iron–doxorubicin complex [6]. While HPLC methods have been developed for determining the parent compound I [7–10] and its one-ring open intermediates and II [8,11], the low peak wavelength and low molar absorptivity ($\epsilon_{205\,nm} \sim 2000\ M^{-1}\ cm^{-1}$) [12] of II limits the use of optical absorbance detection for the determination of II in biological fluids at a



dexrazoxane (I) (II)

Fig. 1. Dexrazoxane (I) and its metal-ion-binding rings-opened hydrolysis product (II).

[1] Dexrazoxane is the generic name of ICRF-187 and ADR-529. ADR-925 is the in-house (Adria Laboratories) name of the dexrazoxane hydrolysis product.

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wavelength where many other compounds strongly absorb. The functional groups present on II are also likely to be difficult to derivatize.

Because II is a strong metal-ion chelating agent [4], it was decided to take advantage of this property to determine II as its metal complex. Terbium(III), which is inherently fluorescent, forms a strong complex with EDTA (stability constant $K_S \sim 10^{18}\ M^{-1}$) [13], and thus also seemed likely to form a strongly bound fluorescent complex with II that could be chromatographed. The fluorescent properties of terbium, gadolinium, eruopium and other metal-ions have been used before in analytical determinations [14–17]. This study describes a method for the determination of II as its terbium(III) complex by reversed-phase ion-pairing fluorescence-detection HPLC that is far more sensitive than UV-Vis absorbance detection; that is far less prone to interferences from biological substances that absorb at low wavelengths; and which requires little pretreatment of the sample.

2. Experimental

2.1. Materials

I and II were a gift from Adria Laboratories (Columbus, OH, USA), HPLC grade methanol was from Mallinckrodt (Mississauga, Canada), 1-heptanesulfonic acid (sodium salt) was from Sigma (St. Louis, MO, USA), terbium(III) chloride hexahydrate and europium(III) chloride hexahydrate were from Aldrich (Milwaukee, WI, USA) and gadolinium (in 1% nitric acid) was from Sigma.

2.2. Methods

The HPLC analysis was conducted using a Varian (Varian Canada, Mississauga, Canada) 9010 pump, a 9050 optical absorbance programmable wavelength detector, Varian Star integration software; a Shimadzu RF-551 HPLC fluorescence detector (Fisher Scientific, Edmonton, Canada); and a Rheodyne injector with a 10-μl loop on a Waters (Millipore, Milford, MA, USA) 10 μm μBondapak C_{18} (300 × 3.9 mm I.D.) reversed-phase column. The optical absorbance and the fluorescent detectors were linked in series in the flow system and their voltage outputs were detected and recorded simultaneously on the computer-controlled two-channel data collection system. Spectrofluorometric studies were carried out in 1 × 1 cm cells on a thermostated Shimadzu RF5000U spectrofluorometer (Tekscience, Oakville, Canada). For the control and calibration experiments, the metal-ion complexes were made by adding stock aqueous solutions of terbium(III) and II together. In the case where II was determined in blood plasma, the proteins were first precipitated from 22.5 μl of plasma by the addition of 8.3 μl of 6 *M* HCl. This was followed by careful neutralization to pH 7.5 with 9.6 μl of 5 *M* NaOH. A 1.7 molar excess of terbium(III) was then added to form the complex. This mixture was then centrifuged for 5 min at 16 000 × *g* and the supernatant was injected without further treatment. The heparin-treated human plasma was separated from the blood cells by centrifugation for 10 min at 600 *g* at 4°C and stored at 4°C. The statistical analysis was carried out using SigmaStat (Jandel Scientific, San Rafael, CA, USA).

3. Results and discussion

3.1. Spectrofluorometric studies

Preliminary experiments were conducted on the spectrofluorometer to determine the optimum excitation and emission wavelengths of the fluorescent metal-ion complexes. The Tb(III)–II complex (10 μM of the 1:1 complex in water) displayed a peak in the emission spectrum at 544 nm that was at a maximum at an excitation wavelength in the range 200–205 nm using 20-nm bandwidths. Terbium(III) alone in water displayed a nearly identical emission spectrum, which was close to that previously described [18,19]. Under similar conditions, the europium(III)–II complex exhibited a somewhat weaker emission peak at 587 nm upon excitation

at 188 nm, similar to that of europium(III) alone [18].

3.2. Chromatographic analyses

In order to chromatograph the Tb(III)–II complex on the reversed-phase column, it was found necessary to include the ion-pairing reagent 1-heptanesulfonate (sodium salt) in the eluent. Using fluorescence detection, as shown in Fig. 2A, Tb(III)–II cleanly eluted with a retention time of 4.6 min using an isocratic eluent containing 50% (v/v) methanol and 50% (v/v) 4 m*M* aqueous 1-heptanesulfonate. The pH of the 1-heptanesulfonate solution was 6.7. The same peak was also simultaneously detected by its absorbance at 205 nm (Fig. 2B). The linearity of the integrated peak areas (area count) with the quantity of the sample injected on the column is shown in Fig. 3. Excellent linearity ($n = 9$, $r = 0.999$ for both) was observed for both fluorescence and absorbance detection over a range of 0.05 to 40 nmol of Tb(III)–II complex that was preformed in a 1:1 ratio. The slope of the plots with their standard error, as determined by linear least squares analysis, was $(9.86 \pm 0.17) \cdot 10^5$ and $(0.50 \pm 0.01) \cdot 10^5$ area counts/nmol using fluorescence and absorbance detection, respectively. The intercepts of both plots were close to zero within their standard errors, with values of $(4.3 \pm 2.9) \cdot 10^5$ and $(0.02 \pm 0.01) \cdot 10^5$ area counts for fluorescence and absorption detection, respectively. The fluorescence detection limit, defined as a signal-to-noise ratio of two, was 25 pmol of Tb(III)–II. The detection limit for absorbance detection at 205 nm was 500 pmol or 20 times larger. It is our experience from analyzing tissue supernatants, plasma, and cell suspensions by absorbance detection of II, that the limit of determination is typically increased to *ca.* 7000 pmol due to presence of biological substances that co-elute with an absorbance at 205 nm.

Experiments employing fluorescence detection were also conducted at Tb(III)/II ratios ranging from 1:1 to 5:1 to test whether the Tb(III)–II complex was stable on the column under the elution conditions used, and also to determine the effect, if any, of a large molar excess of terbium(III). Excess terbium(III) had no effect on the determination of II, as a slope of a plot ($n = 8$) of the quantity of Tb(III)–II *vs.* the Tb(III)/II ratio was zero within one standard error. When terbium(III) alone was injected onto the column, it did not elute under the isocratic conditions used. All of the terbium(III) bound to the column material could, however,

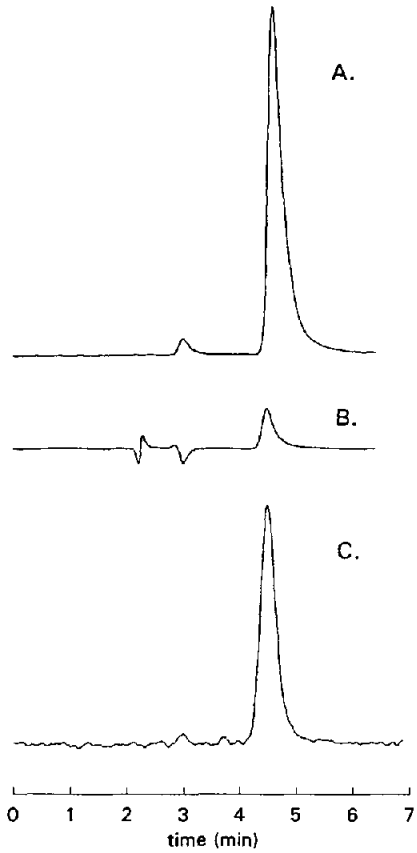

Fig. 2. HPLC of the 1:1 complex Tb(III)–II (10 nmol in water) simultaneously using: (A) fluorescence detection (excitation and emission wavelengths of 200 and 544 nm, respectively) and (B) absorbance detection (205 nm). (C) Chromatogram using fluorescence detection (as in A above, except that the gain was 8 times higher) of the Tb(III)–II complex (0.82 nmol of II and 1.38 nmol of Tb(III)) present in the supernatant of HCl-precipitated plasma (treated as described in the experimental section). The Tb(III)–II complex was eluted isocratically from the C_{18} reversed-phase column with an eluent consisting of 50% methanol and 50% aqueous 4 m*M* solution of the ion-pairing reagent sodium 1-heptanesulfonate.

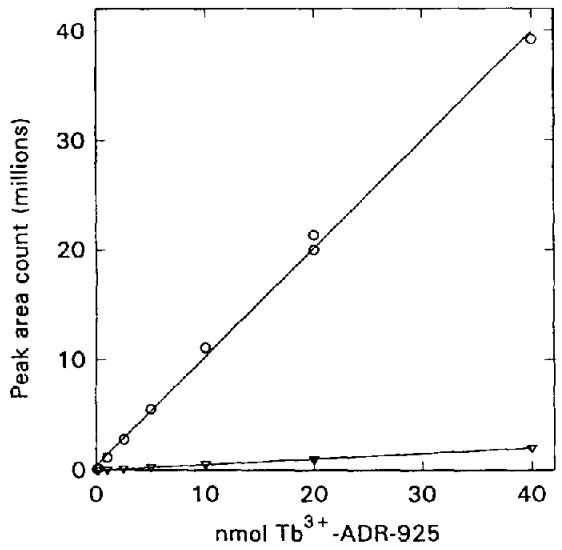

Fig. 3. Plot of the integrated peak-area count *vs.* the quantity of the Tb(III)–II complex by HPLC determined using: (○), fluorescence detection; and (▽), absorbance detection. The straight lines are linear least-squares calculated. Other conditions are as in Fig. 2.

be eluted with repeated injections of II or by rinsing the column with 10 m*M* EDTA for 10 min followed by a rinse with 500 μM EDTA for a further 40 min. EDTA, which could potentially interfere with the analysis, was removed by rinsing the column with methanol overnight.

Experiments were also carried out to see if either Gd(III)–II or Eu(III)–II offered any advantage over terbium(III). Using an excitation wavelength of 280 nm and an emission wavelength of 316 nm [14] for the Gd(III)–II complex, with the same detector settings and column conditions, Gd(III)–II eluted with a retention time of 4.5 min, but with an integrated peak area that was only 2.6% of that found for Tb(III)–II. The sensitivity of the Eu(III)–II complex to fluorescence detection (excitation 394 nm and emission 790 nm) [18] was less than 0.5% of that found for the Tb(III)–II complex, and thus neither of these metal-ions were investigated any further.

Experiments were also carried out to see if II could be determined in blood plasma. II (3.3 m*M*) was added to blood plasma and a 1.7-fold excess of terbium(III) was added to the HCl-precipitated and NaOH-neutralized plasma to form the Tb(III)–II complex. The supernatant of the centrifuged sample was diluted 20-fold for HPLC analysis. Essentially full recovery was obtained, as the amount of II recovered was determined to be 107 ± 3% ($n = 3$) of that initially present. Due to interferences from plasma components, it was necessary to dilute the supernatant of the precipitated plasma before analysis in order to obtain a single cleanly eluting peak. As I was previously shown to form a weak complex with copper(II) [6], mixtures of I and terbium(III) were examined for complex formation. No detectable complex was observed by HPLC. Similarly, the one-ring open (methyl group end) hydrolysis product of I [11] did not form an HPLC-detectable complex. Thus, the presence of the parent drug and its intermediates should not interfere with the determination of II. At 25°C in dilute NaOH, I undergoes a base-catalyzed hydrolysis to II with a $t_{1/2}$ of 30 min [12]. Thus the base-catalyzed conversion of I to II by NaOH could be used as the basis for a sensitive terbium(III)-based fluorescence determination of the parent drug as well. This is a much simpler method than the electrochemical detection column-switching procedure that has been previously used [9].

The hydrolysis of I (Fig. 1) under physiological conditions has been studied by HPLC [11] and spectrophotometrically [12]. The hydrolysis of 3.7 m*M* I at 37°C was followed in plasma titrated to pH 7.39 with Tris-HCl and, for comparison, in Tris buffer (80 m*M* Tris, 60 m*M* NaCl) of the same pH. The loss of I from the incubation mixture was followed by HPLC using absorbance detection at 215 nm, with the gradient previously described [11]. The appearance of II was followed by its determination as the Tb(III)–II complex under isocratic conditions. After 28 h the concentration of I had decayed to 0.46 and 0.18 m*M* in the control and in the plasma, respectively. The concentration of II, as determined by fluorescence detection of its Tb(III)–II complex, was 2.8 and 3.9 m*M* in the control and plasma, respectively. These results indicate that I is hydrolyzed faster in plasma than in buffer. Further studies to characterize this

hydrolysis are underway. In conclusion, this study has shown that the metal-ion binding drug II can be pre-column derivatized by forming a complex with the fluorescent metal-ion terbium(III). The complex formed can be chromatographed by HPLC and detected at low levels by its fluorescence emission at a wavelength where biological substances are unlikely to interfere. The methods developed here that use a fluorescent metal-ion to quantitate the metal-ion complexing drug II might also be applicable to the determination of other metal-ion complexing drugs.

Acknowledgements

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