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Chromatographic and spectroscopic characterization of sulphur-bound dimetridazole and ronidazole derivatives*

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Abstract: The 5-nitroimidazoles, dimetridazole and ronidazole, two important veterinary drugs, were reacted under reductive conditions with the sulfhydryl-containing substrates cysteine and glutathione to yield 5-amino-4-*S*-substituted imidazoles. After purification by reversed-phase liquid chromatography (RP–LC), the four adducts were characterized by RP–LC with photodiode array detection using conditions where their parent drugs were not eluted from the column. Structural identification was conducted by spectroscopic techniques, mainly 1-dimensional and 2-dimensional NMR. While the dimetridazole adducts were found to be monosubstituted at the C-4 position, the two ronidazole products contained two units of the sulfhydryl substrate, located at the C-4 and C-6 positions.

Keywords: *Dimetridazole; ronidazole; nitroimidazoles; RP–LC; nuclear magnetic resonance; cysteine; glutathione.*

Introduction

The group of 5-nitroimidazoles contains well known veterinary drugs that have gained considerable economic importance for their use as antimicrobials in food-producing animals. However, it has been recently demonstrated that some of their metabolic intermediates may covalently bind to tissue macromolecules to form non-extractable residues [1, 2]. Although the levels of drug residues in foodstuffs are usually set by regulatory agencies on the basis of the toxicity of the parent drug and its metabolites, tolerances for persistent drug residues such as those formed by the nitroimidazoles are difficult to determine due to the lack of toxicity data on these compounds. The 1989 Joint FAO/WHO Expert Committee on Food Additives (34th Meeting, Geneva, 1989) was unable to establish ADI (Acceptable Daily Intakes) for a number of 5-nitroimidazoles, with the exception of ronidazole. Similarly, no MRL (Maximum Residue Levels) were allocated because of insufficient information with respect to biological disposition and chemical and toxicological characterization of the metabolites and adducts. Thus, the development of an assay for bound residues, whether for a toxic or non-toxic marker residue, would be of great value to regulatory agencies in determining compliance with residue regulations.

While the structural identity of the nitroimidazole bound residues remains unknown, their formation is generally believed to arise from an interaction between reactive intermediates formed during the metabolic reductive process necessary for drug activation and sulfhydryl-containing substrates. This was demonstrated to be predominant in the binding of ronidazole with microsomal proteins [3].

The preparation of reference compounds bearing a close structural similarity to these bound residues was envisaged for use during metabolic and toxicological studies. These investigations focused on the preparation, purification and characterization of adducts formed between the ubiquitous sulfhydryl-containing compounds cysteine (CYS) and glutathione (GSH), and the 5-nitroimidazoles dimetridazole (DMZ; **1**) and ronidazole (RNZ; **2**).

Experimental

Instrumentation

All analytical LC separations were performed using a Waters 600E Multisolvent

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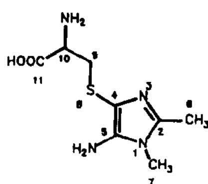
Delivery System coupled to a Waters 900+ Photodiode Array Detector and a Waters 700 Satellite WISP Autoinjector. Data were accumulated and stored on a NEC APC IV Powermate 2 that used Powerline and Waters 990 software for data processing. All separations were monitored over the range 200–400 nm. Optical rotations were measured on a Perkin–Elmer Polarimeter 141 using the sodium D line. All NMR spectra were recorded at 300 K on a Bruker AM400 spectrometer equipped with an Aspect 3000 computer and process controller that used DISNMR version 870101. Standard microprograms from the Bruker Software Library were employed. The ^1H–^1H COSY experiments used N-type phase cycling with a 45° mixing pulse. The long range experiments used an 80 ms delay. The ^1H–^{13}C COSY spectra were obtained using composite phase decoupling with polarization transfer from ^1H to ^{13}C. The raw data were zero-filled in F1 prior to FT, the sine-bell window function being used in both F1 and F2. A proton relaxation delay of 5 s was used. EI and CI mass spectra were measured on a Finnigan-MAT 4610B mass spectrometer using a direct exposure probe (DEP). Fast atom bombardment (FAB) mass spectra were obtained on a VG ZAB 2F at a resolution of 2000, a mass range of 100–950 in 5 s, an accelerating voltage of 7000 V, an M-Scan FAB source and using Xe at 10 kV. "Magic bullet" was used as the matrix.

Materials and methods

Dimetridazole, cysteine, glutathione and sodium dithionite (78%) were purchased from Sigma Co. (St. Louis, MO, USA). Ronidazole was obtained from MSD-AGVET (Merck Frosst, Kirkland, Qué.). Ammonium hydroxide and hydrochloric acid (Baker) and phosphoric acid (88%) (BDH Chemicals) were analytical reagent grade. Ammonium dihydrogen phosphate was HPLC reagent grade (Baker). Biorad AG 50W-X2 Strong Cation (Biorad Labs., Richmond, CA, USA) exchange resin was used. All solvents used were HPLC grade. Analytical LC separations were made on a Brownlee 5-μm Spheri-5 Phenyl column (250 × 4.6 mm i.d., Brownlee Labs., Santa Clara, CA, USA) with a mobile phase consisting of 0.01 M ammonium dihydrogen phosphate, pH 3.6. The flow rate was 2 ml min^{-1} and the column was maintained at ambient temperature. Preparative separations were made on a Waters 5-μm μ-Bondapak C-18 column (300 × 7.8 mm i.d., Waters, Milford, MA, USA) with a mobile phase consisting of water–acetonitrile (92:8, v/v) for the purification of adduct **3**. Purification of the other adducts necessitated adjustments of the amount of acetonitrile. Fractions containing the product were pooled and freeze-dried.

Synthesis of adducts

The adducts were prepared according to the procedure of Wislocki *et al.* [4]. In a typical



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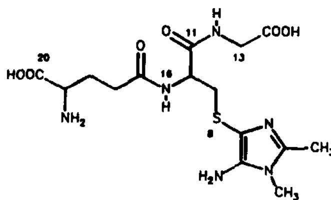

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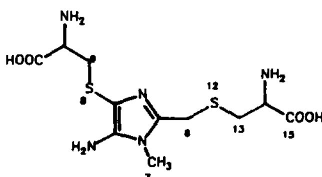

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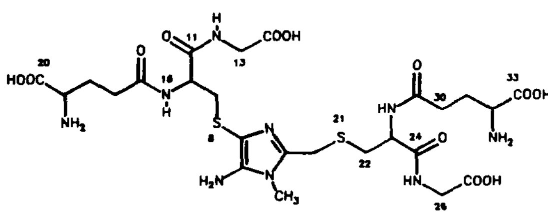

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experiment, sodium dithionite (11.16 g, 0.05 mol) and cysteine (5.6 g, 0.05 mol) were added to a solution of dimetridazole, **1** (1.41 g, 0.01 mol) in water (200 ml), which was adjusted to pH 9.3 with 0.1 N ammonium hydroxide. After stirring at room temperature for 10 min, the reaction mixture was adjusted to pH 3 with concentrated hydrochloric acid and then placed on a strong cation exchange column. Elution with 0.1 N ammonium hydroxide afforded the crude adduct which was crystallized from acetone–water to give the partially purified adduct. Preparative chromatography using the conditions described above afforded adduct **3** in 78% yield (purity by HPLC 98%): m.p. 210–220°C (dec.); $[\alpha]_D$ – 82° H_2O (c 0.76); MS, *m/z* (%). EI: 230 (M^+, 5), 167 (19), 143 (28), 111 (12). FAB: 231 [M^+ + 1].

Adducts **4**, **5** and **6** were prepared in a similar way, using the appropriate starting materials at the stated molar ratios. Some modifications relating to the crystallization solvent were necessary.

Adduct **4**. Yield 58%; purity (HPLC), 97%; m.p. 166–169°C (dec.); $[\alpha]_D$ – 20° H_2O (c 0.82); MS, *m/z* (%). EI: no molecular ion, 215 (5), 128 (8), 111 (12), 85 (70). FAB: 417 [M^+ + 1]. *Adduct* **5**. Yield 68%; purity (HPLC), 94%; m.p. 205°C; $[\alpha]_D$ – 29° H_2O (c 0.17); MS, *m/z* (%). EI: no molecular ion, 270 (2), 208 (4), 161 (8), 75 (10), 44 (100). FAB: 350 [M^+ + 1]. *Adduct* **6**. Yield 47%; purity (HPLC): 98%; m.p. 170–174°C (dec.); $[\alpha]_D$ – 25° H_2O (c 0.49); MS. EI: no molecular ion, no major fragments. FAB: 722 [M^+ + 1].

Results and Discussion

The veterinary drug ronidazole (**2**) has been shown recently to undergo nucleophilic substitution at the C-4 position by a sulphur nucleophile such as cysteine when placed under reductive conditions [4]. This, combined with the ubiquitous nature of cysteine residues in biological macromolecules, indicated that adequate model compounds for persistently bound drug residues may be obtained from relatively small sulfhydryl-containing substrates. Thus, using the conditions described by Wislocki *et al.* [4], dimetridazole (**1**) and ronidazole (**2**) were each reacted with cysteine and the cysteine-containing tripeptide glutathione to afford adducts **3–6** (Fig. 1). The formation of these reduced nitroimidazole adducts has been explained through the formation of an intermediate nitroso derivative acting as an acceptor in a Michael-type addition [4].

The adducts were purified by preparative LC using conditions that avoided the use of buffers to eliminate the subsequent time-consuming ion-exchange chromatography step that was found to induce degradation of the adducts, particularly of adduct **4**. The mobile phases

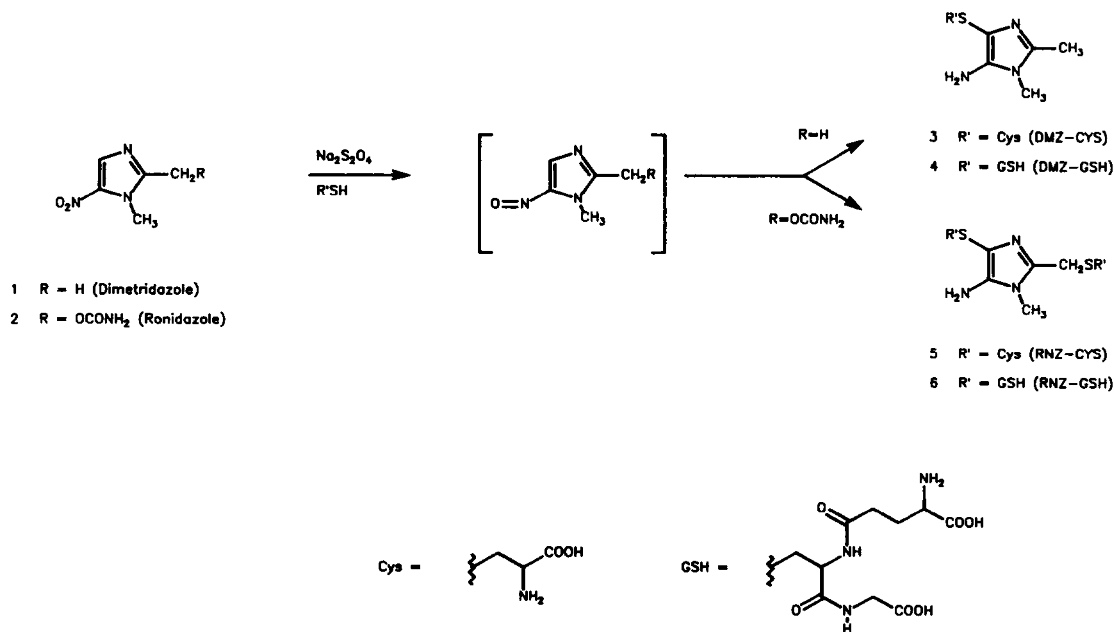

Figure 1
Synthetic scheme for the preparation of DMZ and RNZ adducts.

consisted of water containing a small percentage of acetonitrile. The level of organic modifier was adjusted to elute the product within 20 min of injection and to allow for sufficient separation from the minor constituents. The cysteine adducts **3** and **5** were obtained in slightly higher yields (78 and 68%, respectively) than their glutathione analogues **4** and **6** (58 and 47%, respectively).

LC analysis

LC analysis of the adducts using previously reported conditions [4] (0.01 M NaH_2PO_4, pH 3.6) gave chromatograms with peaks that tailed considerably on conventional octadecylsilyl reversed-phase columns (Brownlee RP-18 and μ-Bondapak). Furthermore, the glutathione adducts **4** and **6** eluted very close to the solvent front under these conditions. The use of a "phenyl" column (Brownlee Spheri-5 Phenyl, 4.6 × 250 nm) was found to reduce significantly the tailing observed with the C-18 columns and to decrease the mobility of the RNZ adducts. Modification of the mobile phase to 0.01 M ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 3.6 was also found to improve peak shape. The chromatograms (detection at 254 nm) in Fig. 2a–d were obtained using these new conditions and show the peaks for the crude adducts prior to purification by preparative LC. The adducts all eluted within 15 min and were well resolved from each other as demonstrated in Fig. 3, which shows the chromatogram for a mixture of the four adducts. Both ronidazole adducts **5** and **6** eluted faster and had better peak shape than their dimetridazole analogues. This is undoubtedly a reflection of the increased hydrophilic character due to the presence of two terminal amino acid units in each compound. The parent drugs, dimetridazole and ronidazole, did not elute from the column under these conditions.

The emergence of LC with photodiode array detection (PDAD) has proven to be an effective tool for the rapid characterization of compounds by a combination of chromatographic retention parameters and spectral data [5]. Moreover, this technique allows detection of structurally related compounds or compounds with similar chromophores under less than optimized chromatographic conditions. The development of the synthetic chemistry of metabolic pathways is a key element in these investigations and as such, a rapid method for the identification of known components in fermentation broths is of particular importance. The UV spectra measured at the apex of the four major peaks in Fig. 3 were obtained with PDAD. In these cases, visual inspection allows unequivocal differentiation between the four adducts. The chromatographic and spectral data obtained on the purified adducts, chromatographed individually, are summarized in Table 1. The two RNZ adducts are characterized by double-maxima UV spectra in the 220–300 nm region while their DMZ analogues feature single-maximum ones. Of

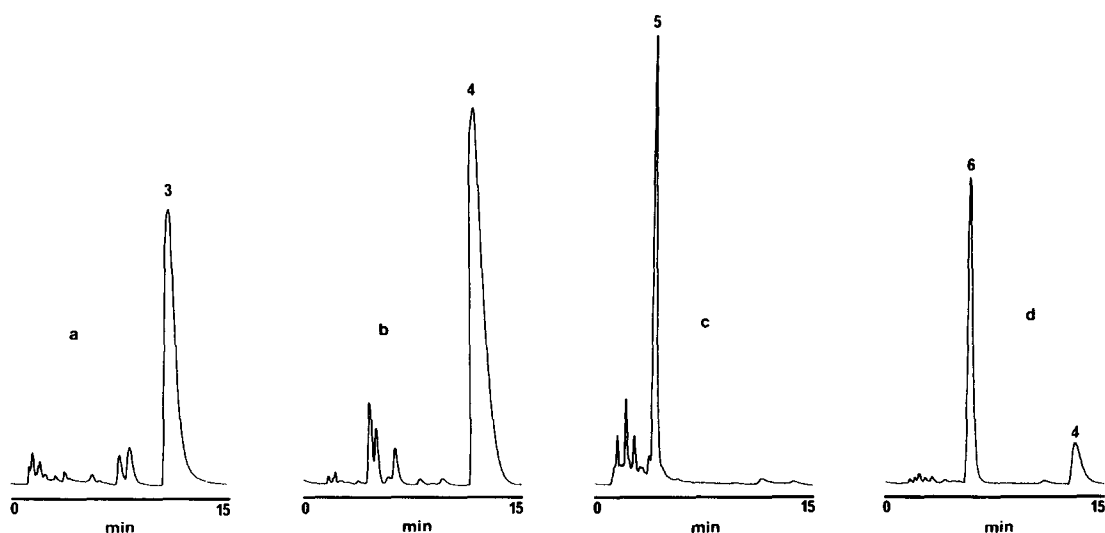

Figure 2
Chromatograms of (a) DMZ–CYS (**3**), (b) DMZ–GSH (**4**), (c) RNZ–CYS (**5**) and (d) RNZ–GSH (**6**) detected at 254 nm.

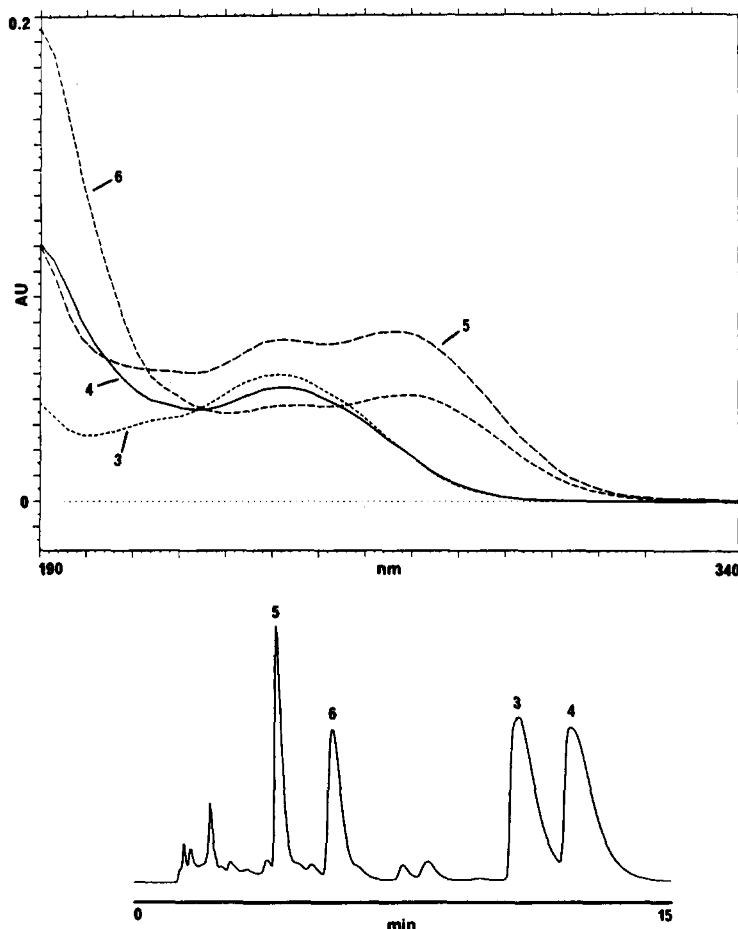

Figure 3
Chromatogram and UV spectra obtained from a mixture of adducts **3–6** using LC with PDAD.

Table 1
Chromatographic and spectral data for the DME and RNZ adducts from LC–PDAD*

Adduct	Retention time (min)	UV data, λ_{max} (nm)
DMZ-CYS (**3**)	10.11	242 (237)†
DMZ-GSH (**4**)	12.44	243
RNZ-CYS (**5**)	4.04	240, 265 (245)†
RNZ-GSH (**6**)	5.62	242, 268

*LC–PDAD: liquid chromatography with photodiode array detection.
†Value in parentheses are take from ref. 4.

interest to note is that the UV maxima observed in these investigations for the two cysteine adducts **3** and **5** differ from the values reported previously [4], particularly in that a single-maximum UV spectrum at 245 nm was reported for **5**. The reason behind these conflicting results is not clear at this point since the change in column support and mobile phase would not be expected to induce such significant variations in the spectra. These UV data were confirmed by obtaining direct UV spectra on purified samples of the adducts that matched the PDAD results. The detection of related constituents in a mixture is one of the major advantages of PDAD and is well exemplified by the identification from its spectral data of adduct **4** in the chromatogram of crude adduct **6** (Fig. 2d). The presence of the monoglutathione adduct **4** along with **6** suggests that the carbamate group undergoes partial hydrogenolysis to the methyl group under these reaction conditions. However, this process does not appear to be significant for the cysteine reaction since only trace amounts of adduct **3** were found in the crude mixture of **5**.

Structural analysis

The structures of the purified adducts were confirmed using a combination of one-

dimensional (1D) and two-dimensional (2D) NMR experiments, which proved particularly helpful for the assessment of the connectivities within the framework. Tables 2 and 3 summarize the carbon-13 and proton chemical shift assignments, respectively, made using a combination of $^{1}H-^{1}H$, DEPT and $^{1}H-^{13}C$ correlation experiments. All proton and carbon-13 spectra were consistent with the expected structures. The proton NMR data for adducts **3** and **5** were in good agreement with the partial data reported elsewhere [4]. The absence of signals corresponding to the C-4 heteroaromatic proton present in the spectra of the parent drugs, as well as the absence of any protonated olefinic carbon resonance as determined by DEPT experiments, confirmed that the substitution reaction occurred at the C-4 position of the imidazole ring at all cases. Overlapping signals were observed in the proton spectrum of adduct **4** and were assigned to the C-13 methylene and the C-19 methine protons on the basis of 2D-COSY experiments. This situation was not observed in the other glutathione-substituted compound **6**. The disubstituted nature of the two RNZ adducts **5** and **6** was confirmed from (a) the disappearance of the carbamate carbonyl resonance and the upfield shift observed for the C-6 signals that has previously been observed in these laboratories, and (b) the integration data from the proton spectra which could only fit disubstituted compounds. The signals from corresponding protons in each of the cysteine units of adduct **5** were found to be overlapped to various degrees. Similar overlaps were observed for the corresponding protons in the glutatione adduct **6**.

Limited information on the structures was obtained from the mass spectra taken in the

Table 2
Carbon-13 NMR data for the adducts DMZ–CYS (**3**), DMZ–GSH (**4**), RNZ–CYS (**5**) and RNZ–GSH (**6**). Obtained in D_2O; values expressed in ppm using TMS as reference

Carbon	3	4	5	6
2	145.08	145.09	143.89	144.21
4	108.12	101.68	108.88	108.30
5	143.25	143.97	141.96	141.84
6	14.58	13.33	29.00	29.19
7	31.91	32.55	31.57	31.59
9	39.79	39.22	38.84	38.47
10	56.96	56.71	56.17*	55.37*
11	175.81	178.82*	174.78	178.08†
13		45.93	34.02	45.28
14		176.53*	55.57*	173.75†
15		—	174.78	—
16		177.53*		176.73†
17		34.07		33.36
18		28.65		28.16‡
19		55.93		56.05
20		173.99*		175.88†
22				34.05
23				54.70*
24				178.06†
26				45.28
27				173.66†
29				176.63†
30				33.36
31				28.03‡
32				56.05
33				175.88†

*†‡ Assignments may be interchanged within the same column.

Table 3
Proton NMR data for the adducts DMZ–CYS (**3**), DMZ–GSH (**4**), RNZ–CYS (**5**) and RNZ–GSH (**6**). Obtained in D_2O; values expressed in ppm using TMS as reference

Carbon	3	4	5	6
6	2.23 s, 3H	2.42 s, 3H	3.80 s, 2H	3.80 s, 2H
7	3.38 s, 3H	3.49 s, 3H	3.45 s, 3H	3.42 s, 3H
9	2.93 dd, 1H	2.92 dd, 1H	2.95 m, 1H	2.83 m, 1H
	3.10 dd, 1H	3.10 dd, 1H	3.10 m, 1H	3.08 m, 1H
10	3.70 dd, 1H	4.37 dd, 1H	3.74 m, 1H	4.25 m, 1H
13		3.71 m, 2H	2.98 m, 2H	3.65 bs, 2H
14		—	3.74 m, 1H	—
17		2.52 m, 2H		2.45 m, 2H
18		2.10 m, 2H		2.10 m, 2H
19		3.70 m, 1H		3.70 m, 1H
22				2.83 m, 1H
				3.08 m, 1H
23				4.25 m, 1H
26				3.65 bs, 2H
30				2.45 m, 2H
31				2.10 m, 2H
32				3.70 m, 1H

s = singlet, dd = doublet of doublet, m = multiplet, bs = broad singlet.

electron-impact (EI) and chemical-ionization (CI) (using methane gas) modes. Adduct **3** was the only one showing a molecular ion fragment in the EI (*m/z* 230, $[M^+]$) and in the CI (*m/z* 231, $[M^+ + 1]$) spectra. The EI spectrum of adduct **4** also featured a major fragment at *m/z* 111 corresponding to the cleavage of the glutathione moiety from the imidazole ring. Molecular weight information was obtained by FAB mass spectrometry where, in all four cases, a fragment corresponding to the protonated molecular ion $[M^+ + 1]$ was observed.

Conclusion

HPLC has been used extensively over the last two decades in pharmaceutical and biomedical analyses due in large part to its high sensitivity and its ability to separate closely related compounds. The LC method presented here reflects these considerations by allowing the rapid identification of related adducts, based on a combination of chromatographic and spectroscopic properties. When used in combination with the rapid spectral acquisition provided by PDAD, this LC method should allow the ready detection of the imidazole chromophore in extracts from biological media.

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