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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION, ISOLATION AND IDENTIFICATION OF 1,2,3-THIADIAZOLE-5-CARBOXALDOXIME GLUCURONIDE IN RABBIT URINE

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SUMMARY

Reversed-phase high-performance liquid chromatography was used to separate and isolate the glucuronic acid conjugate of 1,2,3-thiadiazole-5-carboxaldoxime from urine of rabbits after intravenous injection of the oxime. The conjugate was identified by gas chromatography—mass spectrometry as its trimethylsilylated methyl ester and by nuclear magnetic resonance spectrometry. Additional information was obtained from thin-layer chromatography and high-voltage paper electrophoresis.

INTRODUCTION

Among other oximes 1,2,3-thiadiazole-5-carboxaldoxime (TDA) was tested as an antidote in organophosphate poisoning. To that end its physicochemical and biological properties were studied [1]. During this investigation high-performance liquid chromatography (HPLC) of the urine of rabbits that had been given TDA intravenously revealed that only a negligible amount of unaltered TDA was excreted, whereas at the same time a new UV-absorbing product was found. This paper deals with the separation, isolation and identification of the metabolised TDA.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC equipment was assembled from two pumps (Waters Assoc. Model 6000A), a solvent programmer (Waters Assoc. Model 660), a Valco six-way sampling valve (Model CV-6-UHPa-N60) provided with a 100-μl sample loop

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for analytical purposes or with a 1-ml sample loop for preparative separations, and a variable-wavelength UV detector (Tracor Model 970). In all cases home-made HPLC columns were used. They were packed with LiChrosorb RP-18 according to a modified procedure of Lindner et al. [2]. Before application the performance of the columns was tested. The analytical columns (250 × 5 mm, 5-μm particles) showed 19,000 plates for cumene as test substance ($k' = 1.7$) in the mobile phase methanol—water (5:1, v/v) at a flow-rate of 1 ml/min. The semi-preparative column (250 × 10 mm, 10-μm particles) gave 6000 plates for cumene ($k' = 1.7$) in the same mobile phase at a flow-rate of 2 ml/min.

The following other mobile phases were used. (A) In the case of conjugate analysis the mobile phase was composed of water—methanol—acetic acid (98:2:0.5, v/v). During a chromatographic run a linear gradient was applied to change the mobile phase from 2 to 100% methanol in 30 min. (B) In the case of TDA the mobile phase consisted of water—methanol (4:1, v/v).

Thin-layer chromatography

Silica-gel F254 plates (Merck, No. 11798) were used. The plates were developed with ethyl acetate—water—acetic acid (5:2:2, v/v). The development chamber (20 × 20 × 10 cm) was saturated with the mobile phase. The compounds were detected using UV irradiation (254 nm) on thiadiazole nuclei and *p*-anisidine phthalate (PAP) spray reagent [3] on polyvalent alcohols.

High-voltage paper electrophoresis

The electrophoretic migration was carried out on Whatman No. 1 paper placed in a CAMAG-6100 high-voltage paper-electrophoresis (HVPE) system at pH 3.2 (sodium citrate + hydrochloric acid, 0.1 *M* buffer), 2000 V and 0.03 A.

Mass spectrometry

Electron-impact (EI) and chemical-ionisation (CI; isobutane as reaction gas) spectra were recorded on a VG Micromass 70-70F mass spectrometer using 60 eV electron energy and a source temperature of 200°C.

Gas chromatography

Gas chromatography (GC) combined with mass spectrometry (MS) was carried out on a Varian 1400 gas chromatograph. A glass capillary column (60 m × 0.7 mm I.D.) was used coated with SE-30 as stationary phase. The total ion current served as a detector.

Nuclear magnetic resonance

A Varian XL-100 NMR FT spectrometer was used. The samples were repeatedly dissolved in ^2H_2O and evaporated to dryness in order to remove free hydroxyl protons and were finally run in ^2H_2O solution using sample tubes with an internal diameter of 5 mm. In proton experiments a pulse delay was chosen so as to minimise the solvent peak.

Derivatization reagents

TDA conjugate was derivatized in two sequential steps as described by Compernolle et al. [4]. First the carboxyl group was esterified with diazo-

methane [5], followed by silylation of the hydroxyl groups using trimethylchlorosilane and hexamethyldisilazane [6]. In the case of TDA and the hydrolysed conjugate only the aforementioned silylation reagent was used.

Hydrolysis

The hydrolyses of the conjugate were carried out according to the following methods. (A) Acid hydrolysis: 4 *N* HCl was added to urine (in the ratio 1:1, v/v) and the mixture was set aside at room temperature for 30 min. (B) Enzymatic hydrolysis: a mixture of 2 ml of urine and 3 ml of 0.1 *M* sodium acetate buffer (pH 5.2) was incubated with 0.2 ml of a solution of β-glucuronidase + arylsulfatase (Boehringer, Mannheim, G.F.R.) at 37°C for 3 h.

RESULTS AND DISCUSSION

Urine from rabbits given an intravenous dose of 125 mg of TDA per kg was collected for 24 h. HPLC analysis (solvent system B, see Experimental) of the urine revealed that less than 0.2% of TDA was excreted unchanged [1]. Analysis of the urine using solvent system A (see Experimental) showed the appearance of a new UV-absorbing compound with $k' = 2.5$ as compared with the urine of untreated rabbits (Fig. 1).

The new compound eluted from the HPLC column with a retention time different from that of TDA ($k' = 7$). The addition of acetic acid to the mobile phase proved to be essential to obtain a better separation between the TDA

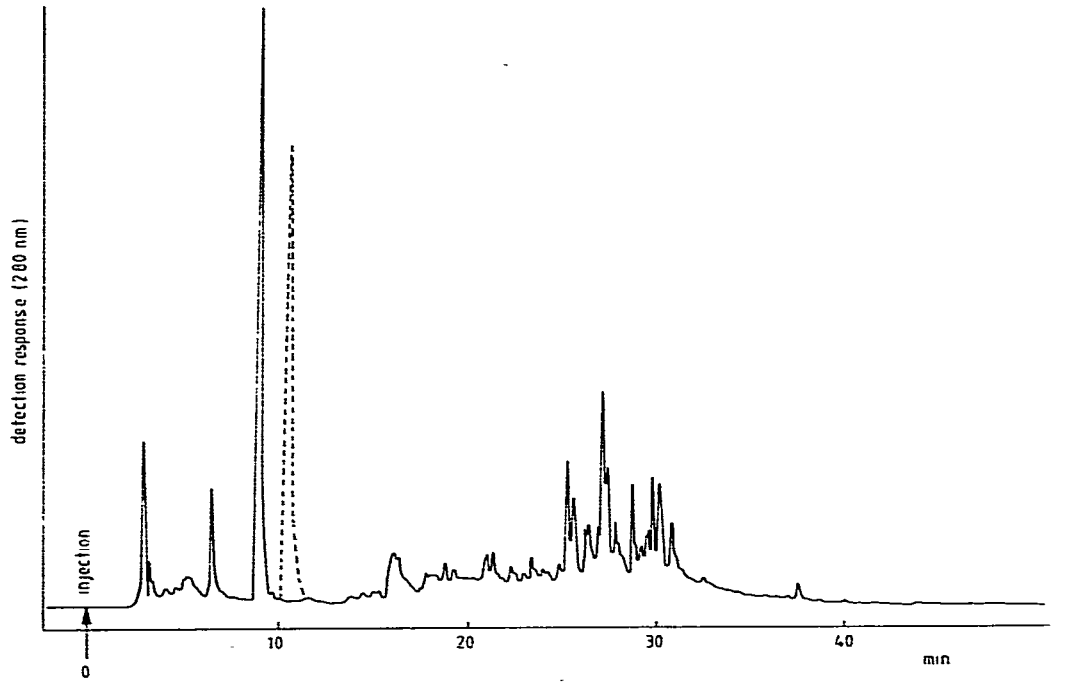

Fig. 1. Typical chromatogram of urine samples from rabbits. ———, urine from untreated rabbits; — — —, TDA conjugate found after intravenous administration of TDA.

TABLE I

THIN-LAYER CHROMATOGRAPHY* OF TDA CONJUGATE AND ITS HYDROLYSATE

Sample	R_F	Characterized as	Detection
TDA conjugate	0.46	—	UV + PAP
Hydrolysed TDA conjugate	0.18	Glucuronolactone	PAP
	0.64	Glucuronic acid	PAP
	0.99**	TDA	UV

*For conditions see Experimental.
**Using ethyl acetate as the mobile phase R_F of TDA is 0.70.

conjugate and the front peaks with k' about zero. On addition of acetic acid the retention time of the TDA conjugate increased considerably. This effect may be explained by assuming the presence of a carboxyl group in the conjugate the dissociation of which decreases on addition of acetic acid. As a result the compound will become more lipophilic and consequently will adhere more strongly to the stationary phase. This assumption of a carboxyl group was sustained by the results of an HVPE experiment in which the conjugate migrated to the anode whereas TDA stayed at the origin.

The HPLC fraction containing the conjugate was compared with a hydrolysed sample (hydrolysis method A, see Experimental) using thin-layer chromatography. The results are presented in Table I. The R_F values and detection results obtained from the hydrolysed conjugate correspond with those of the glucuronolactone, glucuronic acid and TDA, which could be confirmed by the application of GC—MS after silylation of the hydrolysed TDA conjugate. The gas chromatogram is presented in Fig. 2.

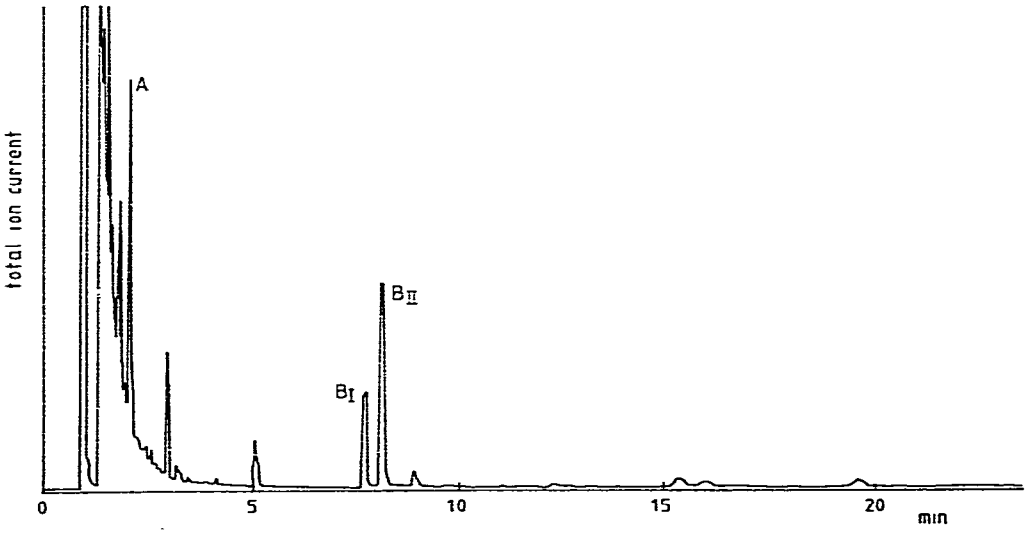

Fig. 2. Gas chromatogram of hydrolysed TDA conjugate after silylation. Column temperature = 200°C. Peaks: A = TMS-TDA; B_I = TMS-α-glucuronolactone; B_{II} = TMS-β-glucuronolactone (TMS = trimethylsilyl).

Table II gives the mass spectra taken from the GC peaks A, B_I and B_{II}. These spectra proved to be identical with those of trimethylsilylated TDA and trimethylsilylated α- and β-glucuronolactone [7], respectively, as was concluded from the use of reference compounds. The presence of the mixture of α- and β-glucuronolactones may be explained by isomerisation of glucuronic acid during the hydrolysis under acid conditions. From these experiments it was concluded that the TDA conjugate is most likely a conjugate of TDA and glucuronic acid.

TABLE II

THE MASS SPECTRA* (EI) OF GAS CHROMATOGRAPHIC PEAKS A, B_I AND B_{II} AS PRESENTED IN FIG. 2

A, B_I and B_{II} were identified as TMS-TDA, TMS-α-glucuronolactone and TMS-β-glucurolactone, respectively (TMS = trimethylsilyl).

Peak A		Peak B_I		Peak B_{II}	
m/e	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)
43	5	45	8	45	8
45	12	73	100	73	100
47	5	74	8	74	9
57	6	75	25	75	18
59	10	103	6	103	7
73	100	133	5	129	6
74	10	147	28	147	20
75	19	217	10	189	5
103	6	230	67	217	15
131	8	231	14	230	73
201	12	232	5	231	18
		259	5	232	6
		287	11	243	7
				245	8
				287	6
				377	7

*Relative intensities ≥ 5%.

Without any derivatization both MS (EI, direct inlet) and GC—MS analysis of the isolated conjugate showed only one substance that could be identified as 5-cyano-1,2,3-thiadiazole. Neither the mass spectrum (EI, direct inlet) nor the gas chromatogram showed any sign of glucuronic acid. This fact indicates that extreme care must be taken in interpreting a cyano derivative as an oxime metabolite [8—10]. Due to thermal instability an oxime conjugate may decompose into a cyano derivative during isolation and analysis.

A stable and volatile derivative of the TDA conjugate was only obtained after methylation followed by silylation, otherwise the conjugate decomposed at high temperatures. In Fig. 3 the gas chromatogram of this derivatized TDA conjugate is presented. The CI-mass spectrum is given in Table III. Besides the protonated molecular ion (*m/e* = 536) the mass spectrum showed the ions *m/e* = 407, 317, 275 and 217, which are characteristic for the glucuronic acid part

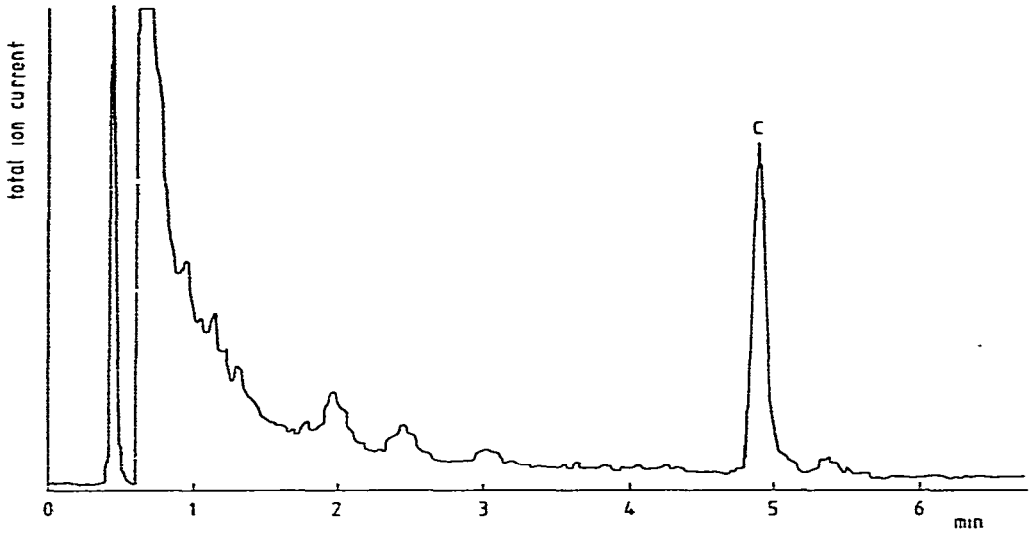

Fig. 3. Gas chromatogram of the methylated and silylated TDA conjugate. Column temperature = 260°C. For the CI-MS data of peak C see Table III.

TABLE III

MASS SPECTRUM* (CI) OF PEAK C IN FIG. 3

m/e	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)
73	26	119	6	187	6	275	10
75	16	130	11	202	6	317	100
89	6	131	5	204	7	318	26
91	43	155	5	215	5	319	13
112	16	159	6	217	19	335	5
114	19	172	6	227	9	407	14
117	5	175	6	245	12	408	6
						536	12

* Relative intensities ≥ 5%.

of the conjugate derivatized as the trimethylsilyl and methyl ester [11]. The TDA part is represented by the ions *m/e* = 130, 114 and 112, which correspond with protonated TDA, TDA ($M^+ - O$) and TDA ($M^+ - H_2O$), respectively.

To carry out ^1H- and ^{13}C-NMR experiments milligram amounts of the TDA conjugate were isolated using the semi-preparative HPLC column (mobile phase A, see Experimental). Volumes of up to 0.5 ml of the urine samples could be injected, giving only a small loss in separation efficiency in comparison with the analytical column. Fig. 4 shows the 100 MHz proton spectrum of the isolated TDA conjugate in ^2H_2O. The numbering of the peaks refers to the corresponding atoms in the formula. The peaks at δ = 9.2 and 8.3 ppm belong to the protons of the thiadiazole ring, which has been verified by means of a reference spectrum of TDA giving the same δ values. The doublet at δ = 5.2 ppm is characteristic for the glucosidic proton 1'. The high value for the coupling

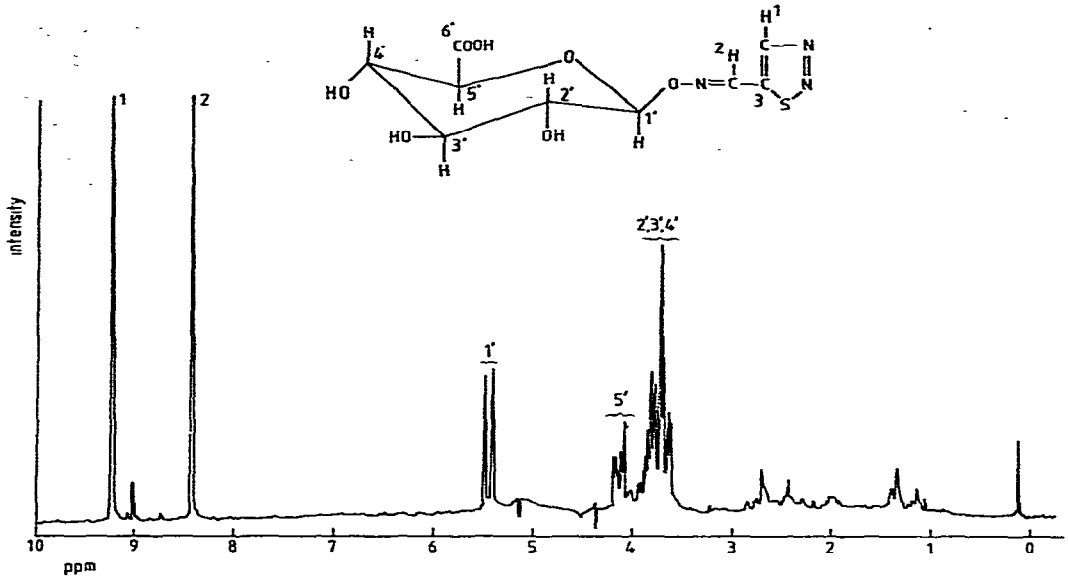

Fig. 4. 100 MHz ^{1}H-NMR spectrum of the isolated TDA conjugate together with the proposed chemical structure. Reference: sodium salt of 3-(trimethylsilyl)propanesulphonic acid.

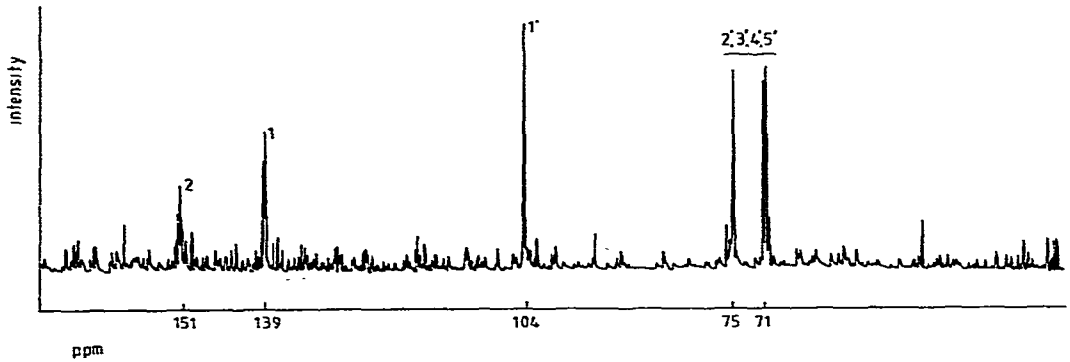

Fig. 5. ^{13}C-NMR spectrum of the isolated TDA conjugate. Numbering is according to chemical structure given in Fig. 4.

constant (J = 7.8 Hz) indicates a *trans* position to the next ring proton 2′ or an axial orientation [12], and points to a β-configuration of the glucosidic linkage [6]. From de-coupling experiments it could be demonstrated that the peak δ = 4 ppm may be ascribed to the proton at site 5′ of the chemical structure. The multiplet at δ = 3.6 ppm belongs to the protons 2′, 3′ and 4′.

Fig. 5 represents the ^{13}C-NMR spectrum of the isolated TDA conjugate. The numbering of the peaks refers to that of the atoms of the chemical structure presented in Fig. 4. The signals at δ = 139 and 151 ppm have been derived from the correspondingly coded carbon atoms (1 and 2) in the thiadiazole part of the conjugate as could be found from reference experiments with TDA. When comparing signals from carbon atoms (1′) in the conjugate and in glucuronic

acid (mixture of α and β form) it was found that the peaks at δ = 92—96 ppm had shifted to δ = 104 ppm in the conjugate linkage. The peaks at δ = 71—75 ppm are comparable with those of glucuronic acid derived from the carbon atoms coded as 2', 3', 4' and 5'. The signals from the other carbon atoms (3 and 6') were too faint to distinguish them from the noise level because of the relative insensitivity for this type of carbon atom. The ^{13}C-NMR spectrum of (α + β)-glucuronic acid is presented in Fig. 6. From the aforementioned NMR experiments it can be concluded that the conjugate consists of TDA and glucuronic acid linked together by a β-linkage. The presence of a β-glucosidic linkage was further confirmed when TDA was liberated from the isolated TDA conjugate on incubation with β-glucosidase.

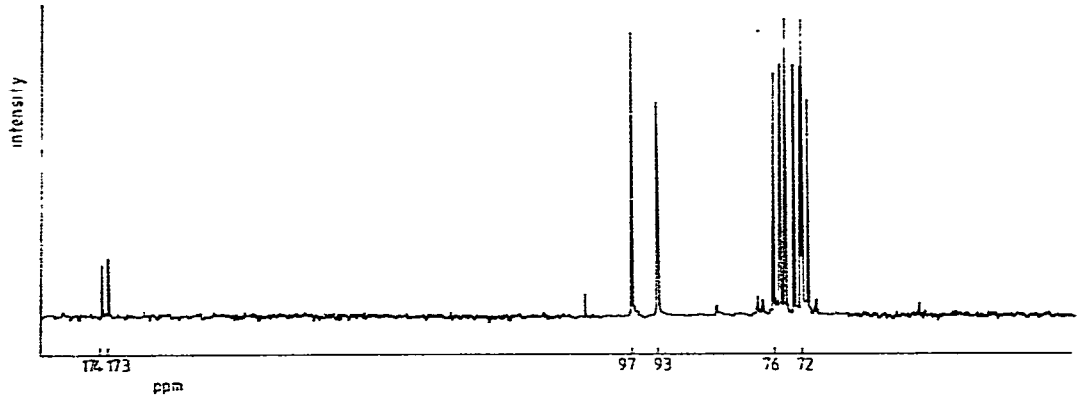

Fig. 6. ^{13}C-NMR spectrum of (α + β)-glucuronic acid.

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