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Gas chromatographic analysis of norcantharidin and related compounds using derivatization to imides*

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Abstract: Derivatization to imides has been studied for determining α,β-diacids or corresponding anhydrides (e.g. norcantharidin) as well as for analysing primary amines by gas chromatography. 2-Naphthylaniline, *n*-heptylamine, cyclohexamine, 2,5-dimethoxyaniline, 2,4,5-trichloroaniline, 3,5-dichloroaniline, aniline and benzylaniline were used as derivatization reagents. ^{1}H-nuclear magnetic resonance and mass spectrometry data were obtained for the norcantharidin *N*-imides synthesized. Factors such as derivatization yields, linearity, reproducibility and sensitivity were evaluated. The influences of reaction temperature and solvent were investigated.

Keywords: *Imide derivatization; gas chromatography; norcantharidin.*

Introduction

Chemical derivatization for gas chromatographic (GC) analysis is a well established technique. Various methods of GC derivatization have been reported in the literature [1–8], but only very rarely has GC imide derivatization been reported. Although Sikka and Rice [9] have determined the herbicide endothal in the aquatic environment by imide derivatization with 2-chloroethylamine, the method of imide derivatization has not been studied in detail. In order to determine serum concentrations of norcantharidin, we have evaluated derivatization with primary amines to imides (Fig. 1). Furthermore, we demonstrate the possibility of using the method of imide derivatization to examine primary amines.

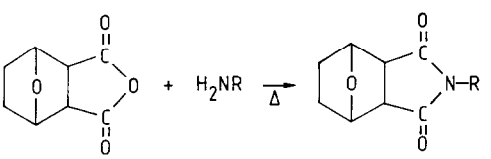

Figure 1
Derivatization of norcantharidin with primary amines.

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Experimental

Materials

Norcantharidin (99%) and the following primary amines were employed: 2-naphthylaniline (99%), aniline (99.5%), benzylamine (99%), 2,5-dimethoxyaniline (98%), 3,5-dichloroaniline (98%), 2,4,5-trichloroaniline (98%), *n*-heptylamine (98%) and cyclohexamine (98.5%). All other reagents and solvents were reagent grade.

Preparation of norcantharidin N-*imides*

A mixture of norcantharidin or endothal (1 mmol), the primary amine (1 mmol) and acetic acid (4 ml) was refluxed at 120°C for about 1 h until all the acetic acid was evaporated. The cooled reaction mixture was rinsed with water to remove remains of acetic acid. The reaction products were recrystallized from cyclohexane–acetone and were weighed to determine the reaction yields. The derivatives were characterized by ^{1}H-nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Analytical derivatization procedure

A mixture of 2–10 nmol of sample (norcantharidin or primary amine), 1 mg reagent (primary amine or norcantharidin) in excess and 1 ml 1% dimethylsulphoxide–benzene was heated at 85°C for 1 h. The reaction temperature was then raised to 130°C and kept for 30 min to take the reaction mixture to dryness. The cooled residue was dissolved in 0.1 ml chloroform for flame ionization detection (FID) or in dioxane for electron capture detection (ECD), depending on the structure of the derivatives. For quantitation, the external standard method was used. The analytical derivatization yields were determined by GC using standards of synthesized norcantharidin *N*-imides. Table 1 shows reaction yields, spectral data and two types of analytical derivatization yields (I) and (II), which were obtained using excess of amine and norcantharidin, respectively.

Analysis of serum samples

Rabbit serum (1 ml) was spiked with norcantharidin (2–10 nmol) and extracted twice with ethyl acetate (2.5 ml) after adjusting the pH to 2 with 2 M HCl. After addition of acetic anhydride (0.1 ml), the solvents were evaporated and 2,4,5-trichloroaniline (1 mg) was added in 1% dimethylsulphoxide–benzene (1 ml). Reaction conditions and sample processing were as mentioned above. The derivative was detected by ECD–GC.

Chromatographic conditions

A Shimadzu GC-7AG gas chromatograph equipped with flame ionization and nickel-63 electron capture detectors was used throughout. The column employed in all studies was a 6.7 m, 0.25 mm (i.d.) fused silica capillary column coated with SE-54. The following chromatographic conditions were used: split injection port temperature, 320°C; total carrier gas nitrogen flow, 40 ml min^{-1}; split ratio, 30:1; make up gas, 20 ml min^{-1}.

Results and Discussion

Analytical derivatization

In order to prove that it is feasible to use derivatization to imides for analysing norcantharidin or primary amines, the reactions were investigated in detail. In both cases linear response curves were obtained. When norcantharidin (2–10 nmol) was reacted

Table 1
Spectral data, analytical derivatization yields and reaction yields for norcantharidin *N*-imides

No.	Imide of	M_W(MS)	NMR(^{1}H,90 MHz,$CDCl_3$)	Analytical derivatization yields (%) I*	II†	Reaction yield (%)
1	2-Naphthylaniline	293	1.64–2.1(4H,m), 3.12(2H,s), 5.1(2H,m), 7.37–8.40(9H,m)	93	96	93
2	Aniline	243	1.42–2.0(4H,m), 3.05(2H,s), 5.0(2H,m), 7.24–7.60(5H,m)	82	87	72
3	Benzylamine	257	1.45–1.93(4H,m), 2.92(2H,s), 4.68(2H,s), 4.95(2H,m), 7.66(5H,s)	103	110	81
4	3,5-Dichloroaniline	311	1.44–2.04(4H,m), 3.1(2H,m), 5.05(2H,m), 7.13–7.53(3H,m)	101	102	64
5	2,4,5-Trichloroaniline	345	1.5–2.1(4H,m), 3.1(2H,d), 5.04(2H,d), 7.36(1H,d), 7.67 (1H,d)	87	100	83
6	2,5-Dimethoxyaniline	303	1.6–2.0(4H,m), 3.1(2H,d), 3.8(6H,d), 5.05(2H,m), 6.75(1H,m), 7.0(2H,d)	98	98	70
7	*n*-Heptylamine	265	0.8–1.9(17H,m), 2.9(2H,s), 3.5(2H,t,J=7.2), 4.9(2H,m)	74	88	85
8	Cyclohexamine	249	1.2–2.2(14H,m), 2.8(2H,s), 3.8–4.1(1H,m), 4.9(2H,m)	47	78	65

* Amines as derivatization reagent.
† Amines as samples.

with an excess of 3,5-dichloroaniline, the regression parameters obtained for the calibration curve ($y = ax + b$) were: $a = 259576$; $b = 4490$; $r = 0.9994$; $N = 5$, and the relative standard deviation (RSD) was 7.4% at the 10 nmol level when samples were analysed in triplicate. When β-naphthylaniline (2–12 nmol) was reacted with excess norcantharidin as a derivatization reagent, the regression parameters were: $a = 3891$; $b = -145$; $r = 0.9979$; $N = 6$, and the RSD value was 5.2% at the 10 nmol level ($N = 3$). These results show that samples in the range of 2–12 nmol can be determined.

Figure 2 shows the chromatogram obtained when different amines were reacted with an excess norcantharidin as derivatization reagent. This result indicates that the derivatization to *N*-imides also allows for the simultaneous measurement of different primary amines.

Effects of reaction temperature and solvent

The following details about the imide derivative reaction should be mentioned. Firstly, it was found that some imide compounds undergo sublimation, e.g. the measured sublimation points of norcantharidin *N*-imides of β-naphthylaniline and 2,4,5-trichloroaniline are 189°C and 170°C, respectively. Secondly, the derivatization reaction to *N*-imides involves dehydration at 130°C which makes the reaction temperature critical with respect to losses of volatile reaction products, which we can expect when amines such as *n*-heptylamine and cyclohexamine are used as reagents. Another consequence is that the derivatization reaction cannot be applied to products which are thermally labile at 130°C. Thirdly, the dehydration temperature appears to be an important factor. Table 2 gives details about the effect of the dehydration temperature in the case of *n*-heptylamine end cyclohexamine, for which the lowest analytical derivatization yields were found (Table 1). From the results it can be seen that a reaction temperature of 120°C results in the best analytical derivatization yields for reaction of norcantharidin with the amines. Fourthly,

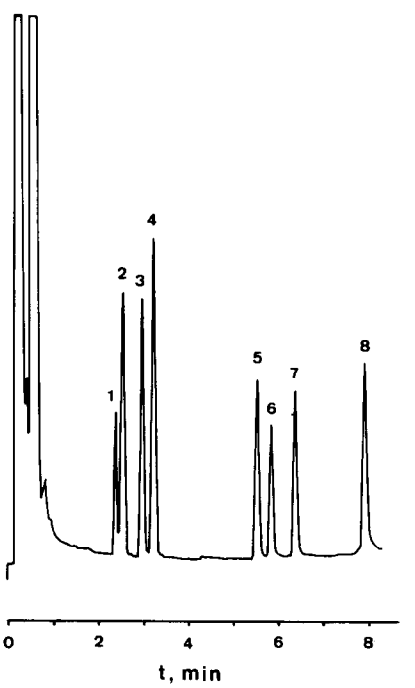

Figure 2
The chromatogram obtained for the determination of a mixture of primary amines using derivatization to norcantharidin *N*-imides. Chromatographic conditions: initial column temperature, 200°C for 1 min; final oven temperature, 260°C; temperature programme, 8°C min^{-1}. Peaks 1–8 correspond to the derivatives of *n*-heptylamine, cyclohexamine, aniline, benzylamine, 3,5-dichloroaniline, 2,5-dimethoxyaniline, 2,4,5-trichloroaniline and 2-naphthylaniline.

Table 2
Effect of dehydrating temperature on the derivatization of norcantharidin

Dehydrating temperature (°C)	Derivatization reagent	Analytical derivatization yield (%)	
		n-Heptylamine	Cyclohexamine
100		<2	<2
120		74	47
140		39	35
160		10	12

from the results given in Table 1, it can also be noticed that the analytical derivatization yields are generally better when amines are reacted with an excess of norcantharidin. Finally, use of dimethylsulphoxide–benzene as solvent system was found to result in higher derivatization yields than glacial acetic acid, which was used by Sikka and Rice [9] for the analysis of endothal. When glacial acetic acid was used as solvent system for the analysis of norcantharidin, we only achieved analytical derivatization yields of approximately 20%.

Analysis of serum concentrations

In order to evaluate whether derivatization to imides can be applied to the determination of drugs in body fluids, we have also carried out determinations of spiked serum samples. The regression parameters obtained for the calibration curve were: a = 319; b = 445; r = 0.9710; N = 5, and the RSD value was 14.3% (N = 3) at the 4 nmol level. The reason for the low precision could be due to the fact that norcantharidin is not efficiently extracted with ethyl acetate.

Conclusion

The derivatization method described above results in a high yield of stable derivatives. The reaction is reproducible and specific for primary amines and α,β-diacids. For the analysis of primary amines, commercially available phthalic anhydride instead of norcantharidin can be considered as a derivatization reagent. More research with respect to standardization is however necessary to make the proposed method analytically useful for precise determination of serum norcantharidin levels.

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