Journal of Chromatography, 189 (1980) 375–388

CHROM. 12,463

EXTRACTIVE ALKYLATION OF 5,2'-DICHLORO-4'-NITROSALICYLANILIDE (NICLOSAMIDE) FOR GAS–LIQUID CHROMATOGRAPHIC ANALYSIS

FREDERICK C. CHURCHILL, II and DAVID N. KU

Vector Biology and Control Division, Bureau of Tropical Diseases, Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Ga. 30333 (U.S.A.)

(Received October 16th, 1979)

SUMMARY

Niclosamide (5,2'-dichloro-4'-nitrosalicylanilide), formulated as the ethanolamine salt, is one of the most effective and widely used molluscicides for the control of snail vectors of schistosomiasis, a parasitic disease afflicting over 200 million people in more than 70 countries. This report details the development of a sensitive, specific analytical method for the evaluation of niclosamide formulations and assessment of the impact of residues on the environment. Efficient (>85%) phase-transfer, N,O-dimethylation of niclosamide and the synthesized 5-deschloro analog internal standard, followed by gas–liquid chromatographic separation and electron-capture detection, permits the determination of as little as 10 ppb* analyte in fortified, stagnant water. Tetrabutylammonium chloride was the phase-transfer agent, while methyl iodide was the methylating agent of choice among four investigated.

INTRODUCTION

Schistosomiasis is a parasitic disease afflicting over 200 million people in more than 70 countries. Among the diseases affecting mankind, its prevalence is second only to malaria[1]. Niclosamide (5,2'-dichloro-4'-nitrosalicylanilide), formulated as the ethanolamine salt, is one of the most effective and widely used molluscicides for the control of snail vectors of schistosomiasis[2]. There is widespread interest in monitoring niclosamide residues in the environment in order to evaluate the effectiveness of different formulations, particularly the slow-release formulations[3], and to assess the impact of niclosamide not only on the vector snails but also on non-target organisms which may share the same habitats[4–6]. Niclosamide has been used as an anthelmintic in human[7,8] and veterinary[9,10] medicine, so that a need exists for good clinical methods of analysis.

Existing assays for niclosamide include colorimetric methods[11–15] and bioassays[15,16]. These methods lack the specificity necessary for precise, accurate results.

* Throughout this article, the American billion (10^9) is meant.

Formation of a volatile derivative is necessary before gas–liquid chromatographic analysis of niclosamide is possible. The stability of alkyl phenyl ethers and of N-alkylamides suggest alkylation as a reasonable approach. Phase-transfer alkylation offers a number of advantages over more traditional alkylation methods.

Theoretical and synthetic aspects of phase-transfer catalysis have been treated in a number of recent reviews[17–20]. Durst *et al.*[21] have used crown ethers as solid–liquid phase-transfer reagents in the formation of phenacyl esters of fatty acids prior to liquid chromatographic analysis. The use of large quaternary ammonium ions for ion-pair extractions is well known[22,23], as is the efficacy of such ions in promoting the alkylation of acidic substances in polar organic solvents[24].

Extractive alkylation has been used in the quantitative determination of a number of organic compounds in physiologic substances (see refs. 25 and 26 and references therein). Analytes determined using extractive alkylation include fatty acids[26], chlorthalidone[27], furosemide[28], hydrochlorthiazide[29], nitrazepam[30], chlonazepam[31], and fluonitrazepam[31], among others.

The formation of the N,O-dimethyl derivative of niclosamide and of a similar internal standard compound using liquid–liquid phase-transfer alkylation has been investigated in the present study. This approach might reasonably be expected to form volatile methyl derivatives which would allow exploitation of the potential electron-capture sensitivity of the *p*-nitrobenzamide moiety. The nature of the phase-transfer alkylation procedure lends itself to sample concentration and cleanup in addition to efficient alkylation. The ultimate goal of this study, then, was the development of a gas chromatography–electron-capture detector (GC–ECD) method which allows specific, precise, and accurate analysis of niclosamide in water and a variety of other sample matrices.

EXPERIMENTAL

Standards

Standard 5,2′-dichloro-4′-nitrosalicylanilide (niclosamide) of 99.4% purity was kindly provided by the Chemagro Agricultural Division of Mobay Chemical Corporation (Kansas City, Mo., U.S.A.)*. Two candidate internal standard compounds, 2′-chloro-4′-nitrosalicylanilide (2′-Cl-4′-NSA) and 5-chloro-4′-nitrosalicylanilide (5-Cl-4′-NSA), were synthesized by a condensation procedure adapted from that of Schraufstatter *et al.*[32].

The 2′-Cl-4′-NSA was prepared by the condensation of salicylic acid and 2-chloro-4-nitroaniline. A 13.8-g quantity (0.1 mol) of the former was added to 17.3 g (0.1 mol) of the latter in a 500-ml standard taper round-bottom flask. The mixture was refluxed in 200 ml of xylene, effecting solution. Phosphorus oxychloride (4.0 ml) was added and refluxing resumed for 3.0 h. The resulting mixture was filtered while warm to yield 28.0 g of air-dried product. Washing sequentially with ethanol, chloroform, and ether yielded 19.8 g of air-dried powder. A 7.4-g portion of this product was recrystallized from 350 ml of xylene to yield 6.4 g of tan microcrystalline product, m.p. 232.0 to 232.5°.

* Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

The 5-Cl-4'-NSA was prepared by condensation of 2-hydroxy-5-chlorobenzoic acid and 4-nitroaniline in a manner analogous to that employed for the formation of 2'-Cl-4'-NSA. The yellow crystalline material resulting from the xylene recrystallization had a melting point of 264.0 to 264.5°.

The N,O-dimethyl derivative of niclosamide was synthesized using phase-transfer alkylation, adapting reaction conditions from several sources[33–35]. A 2.0-g quantity of technical Bayluscide (ethanolamine salt of niclosamide) was dissolved in 50 ml of 4% aqueous sodium hydroxide in a 500-ml screw-cap erlenmeyer flask. To this was added 100 ml of chloroform, 2.5 g of tetrabutylammonium chloride (TBACl) and 10.0 ml of dimethyl sulfate. The two phases were stirred overnight using a magnetic stirrer. The chloroform layer was separated and evaporated. The residue was partitioned between water and ether. The ether layer was washed successively with two portions of 2 *M* ammonia, one portion of 2 *M* sodium hydroxide and two portions of water. The ether was evaporated. Benzene was added and then removed by rotary vacuum evaporation in two successive steps to remove residual water. The residue was triturated with diethyl ether and the 1.1 g of solid product filtered from the mixture. Recrystallization of a 500-mg quantity using isooctane–benzene yielded 400 mg of crystalline N,O-dimethylniclosamide, m.p. 169.5–170.0°, after vacuum drying: nuclear magnetic resonance (NMR) (C^2HCl_3) δ 8.22 (d, 1, J=2.0 Hz, position 3'), δ 7.94 {d, 1, J=7.0 Hz (J=2.0 Hz, meta splitting), position 5'}, δ 7.0 to 7.5 (m, 3, positions 4,6,6'), δ 6.62 (d, 1, J=7.0 Hz, position 3), δ 3.70 (s, 3, O-CH_3), δ 3.36 (s, 3, N-CH_3). The resonances are broader than would normally be expected. The mass spectrum is shown in Fig. 1A.

N,O-Dimethyl-2'-chloro-4'-NSA was synthesized by an analogous phase-transfer methylation procedure. The recrystallized (isooctane–benzene), vacuum dried product has a melting point of 92.6 to 93.0°: NMR (C^2HCl_3) δ 8.17 (d, 1, J=2.0 Hz, position 3'), δ 7.90 {d, 1, J=7.0 Hz (J=2.0 Hz, meta splitting), position 5'}, δ 7.0–7.5 (m, 3, positions 4,6,6') δ 6.5–7.0 (m, 2, positions 3,5), δ 3.72 (s, 3, O-CH_3), δ 3.36 (s, 3, N-CH_3). Again, the resonances are broader than might have been expected. The mass spectrum of N,O-dimethyl-2'-chloro-4'-NSA is shown in Fig. 1B.

N,O-Dimethyl-5-chloro-4'-NSA was prepared analogously to the above dimethyl derivatives. The recrystallized (isooctane–benzene), vacuum-dried product has a melting point of 105.2 to 105.6°: NMR (C^2HCl_3) δ 8.04 (d, 2, J=9.0 Hz, positions 3',5'), δ 7.29 (s, 1, position 6), δ 7.29 (d, 2, J=9.0 Hz, positions 2'.6'), δ 7.21 (d, 1, J=9.0 Hz, position 4), δ 6.66 (d, 1, J=9.0 Hz, position 3), δ 3.59 (s, 3, O-CH_3), δ 3.45 (s, 3, N-CH_3). The resonance peaks were of normally expected sharpness.

Reagents

Tetrabutylammonium chloride (85% pure, remainder *n*-butanol) was purchased from Aldrich (Milwaukee, Wisc., U.S.A.), as was the methyl iodide, methyl toluenesulfonate (methyl tosylate) and methyl methanesulfonate (methyl mesylate). The dimethyl sulfate was supplied by Mallinckrodt (St. Louis, Mo., U.S.A.). Benzene and methylene chloride were glass-distilled solvents, available from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). All other chemicals were reagent grade.

Equipment

Reaction product characterization was performed using a Hewlett-Packard

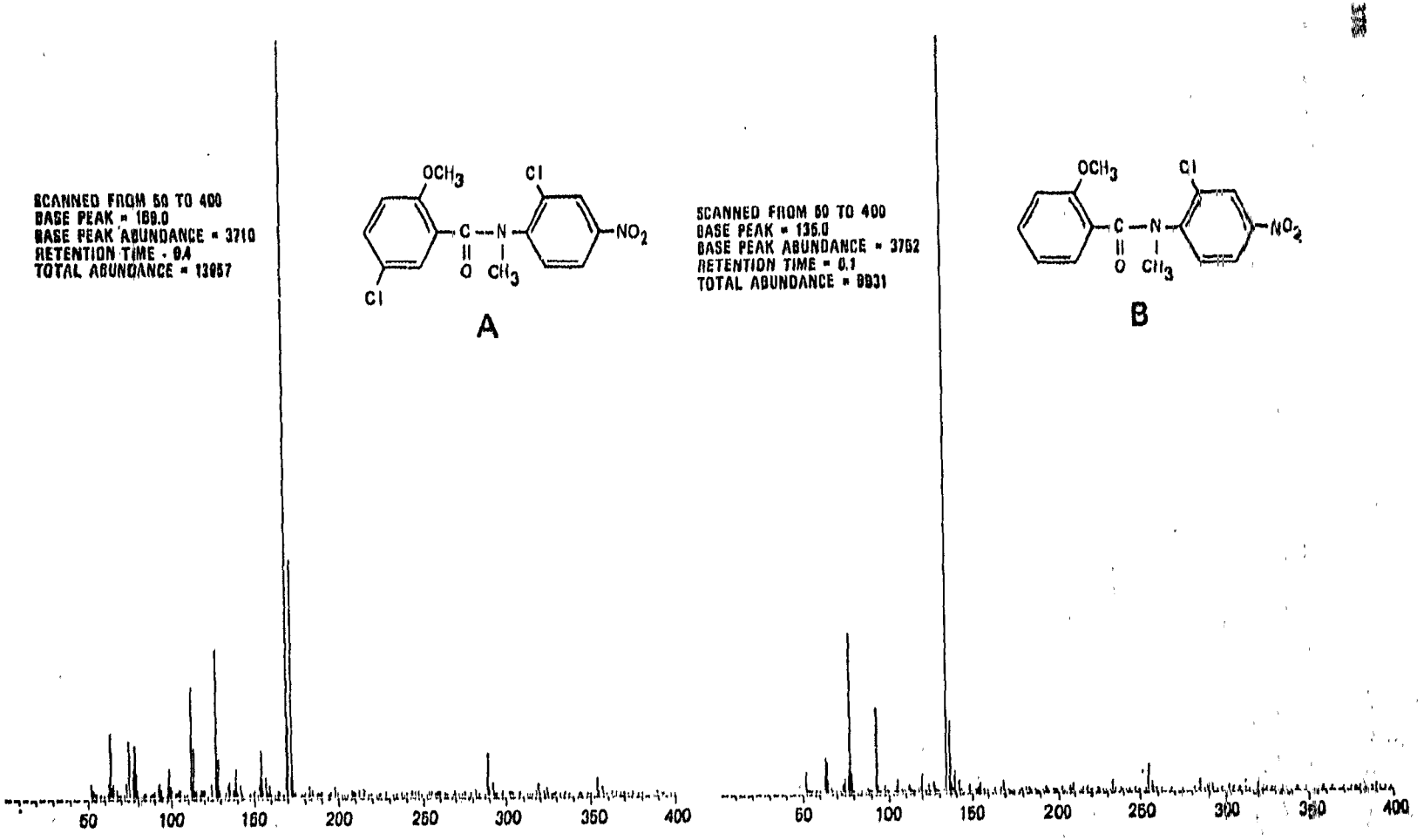

Fig. 1. Electron-impact mass spectra and structures of (A) N,O-dimethylniclosamide and (B) N,O-dimethyl-2′-chloro-4′-nitrosalicylanilide.

Model 5992 gas chromatograph–mass spectrometer–computer system. Studies were performed using 1.83 m × 2.0 mm I.D. glass columns, one packed with 4% OV-101 on 100–120 mesh Chromosorb W HP and a second packed with 5% OV-101 on 100–120 mesh Gas-Chrom Q. A helium flow-rate of 16 ml/min was used. Injector, column, jet separator, and source temperatures were 250, 240, 240, and 150°, respectively. Electron-impact ionization was performed at 70 eV.

A Varian Model 1700 gas chromatograph equipped with an Aerograph scandium tritide detector was utilized for the optimization of reaction conditions and for the gas–liquid chromatographic analysis of samples by the optimized procedure. The column employed was a 1.83 m × 2.0 mm I.D. glass column packed with 5% OV-101 on 100–120 mesh Gas-Chrom Q. The column temperature was 230° while the injection port was held at 260° and the detector at 270°. Nitrogen carrier gas at a flow-rate of 60 ml/min was used. Analog data were recorded on a Varian Model 9176 recorder.

Proton NMR spectra were scanned using a Perkin-Elmer Model R12B spectrometer.

Melting points were obtained using a Fisher-Johns apparatus which had been calibrated using Fisher Thermetric standards.

Hydrolysis of methylating agents under methylation conditions

The rate of disappearance of methyl iodide under the derivatization conditions developed was followed by running proton NMR spectra on aliquots of the methylene chloride layer taken at intervals during a methylation and comparing the intensities of the δ 2.14 singlet. Similar determinations were run for dimethyl sulfate, methyl tosylate, and methyl mesylate by noting the loss of intensity in the ^{1}H NMR spectra of the singlets at δ 3.94, δ 3.68, and δ 3.87, respectively, in methylene chloride. The reaction conditions were those utilizing 10 ml of CH_2Cl_2, 250 mg of TBACl, and 50 ml of 3.0% aqueous NaOH.

Determination of methylation efficiencies using various methylating agents

Methyl iodide. A 1.0-ml volume of standard containing 25.61 μg/ml of niclosamide and 25.16 μg/ml of 2′-Cl-4′-NSA in methanol was added to 50 ml of 3.0% aqueous NaOH in a 125-ml screw-cap erlenmeyer flask. A 10.0-ml quantity of methylene chloride was added, followed by M_1 mg of TBACl in methanolic solution. After the addition of 2 ml of methyl iodide, the flask was equipped with a magnetic stirring bar, capped, and stirred for time T_1. Table I shows the values used for M_1 and T_1.

At the end of the stirring time a separatory funnel was used to isolate the methylene chloride layer. The latter was drained into a 50-ml screw-cap test tube. The aqueous layer was extracted with two 5-ml portions of additional methylene chloride and these combined with the original methylene chloride layer. The solvent was evaporated on a steam bath and 10 ml of benzene added to the residue. After the benzene solution was partitioned with 10 ml of water to remove phase-transfer reagent, a 5.0-ml aliquot of the benzene layer was taken and evaporated to near dryness to azeotrope residual water. A 10-ml quantity of benzene was added followed by 1 to 5 dilution with benzene. This solution was then compared by GC–ECD with dilutions of a standard containing authentic N,O-dimethylniclosamide and N,O-dimethyl-2′-chloro-4′-NSA.

TABLE I

GC–ECD RESULTS FOR PHASE-TRANSFER ALKYLATION EXPERIMENTS USING VARIOUS METHYLATING REAGENTS

In each case 50 ml of 3% aqueous NaOH was used and 25.61 and 25.16 μg of niclosamide and internal standard, respectively, were derivatized. MI = methyl iodide; MS = methyl sulfate; MT = methyl tosylate; MM = methyl mesylate.

Designation	*Derivatization cond.*			*Conversion eff. (%)*	
	Alkylating agent, volume (ml)	*TBACl M_1 (mg)*	*Reaction time T_1 (min)*	*Niclosamide*	*2'-Cl-4'-NSA*
MI-1	MI, 2.0	250	10	47.1	38.2
MI-2	MI, 2.0	250	30	85.7	84.2
MI-3	MI, 2.0	250	45	91.2	88.6
MI-4	MI, 2.0	250	60	95.1	90.9
MI-5	MI, 2.0	250	90	96.1	88.9
MI-6	MI, 2.0	250	120	95.6	88.2
MI-7	MI, 2.0	250	240	94.9	86.4
MI-8	MI, 2.0	250	960	81.9	77.4
MI-9	MI, 2.0	25.0	90	91.0	55.1
MI-10	MI, 2.0	25.0	960	99.7	85.3
MS-1	MS, 1.0	250	5	71.3	48.1
MS-2	MS, 1.0	250	10	78.0	63.6
MS-3	MS, 1.0	250	15	93.2	72.3
MS-4	MS, 1.0	250	30	91.2	87.7
MS-5	MS, 1.0	250	60	95.1	93.2
MS-6	MS, 1.0	250	120	94.2	95.0
MS-7	MS, 1.0	250	240	96.7	93.4
MT-1	MT, 3.0	250	60	97.9	91.3
MM-1	MM, 1.0	250	120	46.0	56.7

Dimethyl sulfate, methyl mesylate, and methyl tosylate. Methylation trials were held using a number of other methylating reagents[36], including dimethyl sulfate, methyl mesylate, and methyl tosylate. Since the sulfate and sulfonate reagents are not particularly volatile, a step was inserted in which 2-aminoethanol was added to destroy excess reagent and the methylene chloride layer extracted with water. The methylene chloride was then evaporated and the conversion efficiency determination continued as outlined above.

Characterization of the methylation products

The methylation products of niclosamide and 2'-Cl-4'-NSA were identified by separate derivatization runs of 500-μg quantities followed by GC–mass spectrometry analysis. The methylated products were compared to authentic N,O-dimethylniclosamide and N,O-dimethyl-2'-chloro-4'-NSA.

Test to establish the possibility of iodide inhibition of niclosamide phase-transfer alkylation

Into a 125-ml screw-cap erlenmeyer flask was placed 10 ml of CH_2Cl_2, 50 ml of 3% aqueous sodium hydroxide, 2.0 ml of 125 mg/ml TBACl in methanol and 2.0 ml of methyl iodide. This mixture was stirred for 90 min. At this point 1.0 ml was added of a solution containing 25.7 μg/ml of niclosamide and 25.8 μg/ml of 2'-Cl-4'-

NSA in methanol. The reaction was allowed to proceed with magnetic stirring. The yellow color was extracted into the CH_2Cl_2 immediately and then disappeared with time. The sample was prepared for GC–ECD analysis as outlined previously. The final dilution was compared with the mixed dimethyl standard to determine conversions.

Extraction efficiency determination for niclosamide and 2'-Cl-4'-NSA

Into a 125-ml screw-cap erlenmeyer flask was placed 1.0 ml of a solution containing 25.7 μg/ml of niclosamide and 25.8 μg/ml of 2'-Cl-4'-NSA in methanol, 10 ml of CH_2Cl_2, 50 ml of 3% aqueous sodium hydroxide, and 25 mg of TBACl. This mixture was capped and stirred magnetically for 3 min. The two layers were separated using a separatory funnel and one 5-ml CH_2Cl_2 rinse. Each was placed in a separate 125-ml erlenmeyer flask. To the organic layer was added 50 ml of 3% aqueous NaOH, 250 mg of TBACl, and 2 ml of CH_3I. To the aqueous layer was added 10.0 ml of CH_2Cl_2, 250 mg of TBACl, and 2 ml of CH_3I. Each was stirred for 90 min. Both samples were prepared for GC–ECD analysis as outlined previously. The final dilution was compared with the mixed dimethyl standard to determine conversions.

Linearity and precision studies

A 100-ml quantity of 251.6 μg/ml of 2'-Cl-4'-NSA in methanol was made up as was a similar quantity of 256.1 μg/ml of 99.4% pure niclosamide standard. Careful and accurate dilution produced solutions containing 25.16 μg/ml of internal standard and 0.0, 5.122, 25.61, and 51.22 μg/ml of niclosamide standard.

Into a 250-ml erlenmeyer flask equipped with a teflon-lined screw-cap were placed 1.0 ml of the fortifying solution, 10.0 ml of methylene chloride, 50 ml of 3.0% aqueous NaOH, and 2.0 ml of 125 mg/ml TBACl in methanol. The sample was stirred rapidly for 1 min. A 2.0-ml quantity of methyl iodide was added followed by moderate stirring for 75 min.

In each case the mixture, after 75 min stirring, was transferred to a 125-ml separatory funnel. The methylene chloride was drained into a 50-ml screw-cap test tube and the solvent evaporated on a steam bath using a boiling capillary. A 10-ml quantity of benzene was added, followed by vortexing. The benzene layer was partitioned against 10 ml of water in the test tube. After separation of the layers, about 8 ml of the benzene layer was transferred by pipet into a 20-ml test capillary. The benzene was evaporated to near dryness on a steam bath using a boiling tube. The residue was diluted to about 10 ml and the resulting solution mixed and diluted 1 to 5 in benzene. About 2 μl of this dilution was injected into the GC–ECD instrument in duplicate for each of the niclosamide concentrations to establish the peak height ratio for each. The peak height ratios were plotted against the total μg of niclosamide in the sample to arrive at the standard curve.

Analysis for niclosamide in technical grade Bayluscide, stagnant water, and urine using methyl iodide

A 30.3-mg sample of technical grade Bayluscide, Chemagro batch number 1124-21, was weighed into a 100-ml volumetric flask and diluted to the mark with methanol. A 5.0-ml aliquot of this solution and a like aliquot of 251.6 μg/ml internal standard were added to a 50-ml volumetric flask and diluted to the mark with meth-

anol. Three 1-ml quantities were derivatized in the same manner as for the standard curve procedure using methyl iodide and were quantified by GC comparison to a calibration curve run concurrently.

Triplicate determinations were made on samples fortified with 25.16 μg of internal standard and 25.61 μg of niclosamide in urine and then in stagnant, dirty water. In these determinations a solvent extraction, prederivatization, sample cleanup was utilized. In this procedure, when utilized for urine cleanup, 25 ml of urine was added to a 125-ml separatory funnel and fortified with internal standard and niclosamide. This was followed by the addition of 0.5 ml of 12 *N* HCl and swirling to mix. The aqueous acid solution was then partitioned with 50 ml of diethyl ether–toluene (50:50). The aqueous layer was drawn off and discarded. A 50-ml quantity of 3.0% aqueous NaOH was added to the separatory funnel and the mixture shaken. The aqueous layer was then drained into a 250-ml erlenmeyer flask. To this was added 10 ml of methylene chloride and 2 ml of 125 mg/ml TBACl in methanol, followed by 1 min of rapid magnetic stirring. The 2.0-ml addition of methyl iodide was made and the derivatization completed as for the calibration standards. The only variance from this procedure was the insertion of a plug of cotton 3 cm long into the delivery tube of the separatory funnel when the methylene chloride was separated from the aqueous portion of the derivatization mixture. This technique broke the minor emulsion in the methylene chloride phase and permitted the collection of a clear methylene chloride layer.

The same procedure was used for the stagnant, dirty water samples except that 50 ml of the stagnant water was taken and no emulsion problems were confronted.

An experiment was run to establish the minimum detection limit for niclosamide in water. A sample with deionized water and one with stagnant, dirty water were each fortified with 2.516 μg of internal standard and 0.5122 μg of niclosamide. Each was analyzed using the above procedure up to the point where benzene is evaporated from the 20-ml test tube. Here the sample was quantitatively transferred using benzene to a benzene-saturated and compressed bed of Celite 3 cm deep in a 1-cm diameter column. The column was eluted with 10 ml of benzene. The eluate was evaporated to near dryness and diluted to about 5 ml with benzene prior to GC–ECD quantitation.

RESULTS AND DISCUSSION

Characterization of the dimethyl derivatives of niclosamide and the two internal standard candidates

The characterization of the N,O-dimethyl derivatives of niclosamide, 2′-Cl-4′-NSA, and 5-Cl-4′-NSA by ^{1}H NMR yielded important information. The spectrum of N,O-dimethyl-5-Cl-4′-NSA was sharp, while the two derivatives with chlorine in the 2′ position exhibited broad peaks. The broadness, which was quite apparent in both methyl peaks of each of these two compounds, is due to restricted rotation about the amide bond. A lower temperature would yield the ^{1}H NMR spectrum of the individual rotamers. The barrier is high relative to that for the 5-chloro compound because chlorine in the 2′ position inhibits the attainment of the coplanarity necessary for the delocalization of electron density into the *p*-nitrophenyl moiety. Therefore, negative charge density delocalization toward the carbonyl occurs, lending ap-

preciable double bond character to the amide bond. The structural similarity of 2'-Cl-4'-NSA to niclosamide in the amide portion of the molecule identifies it as the better of the two internal standard candidates. Its dimethyl derivative is also better separated chromatographically from N,O-dimethylniclosamide on OV-101 than is N,O-dimethyl-5-Cl-4'-NSA[36].

GC–mass spectrometric characterization of the N,O-dimethyl derivatives of niclosamide and the chosen internal standard yielded the mass spectra shown in Fig. 1. In the former the molecular ion is seen at $m/e = 354$ with isotope abundances as expected for the presence of 2 chlorine atoms. The base peak at $m/e = 169$ is due to the fragment formed by amide cleavage. Corresponding peaks in the dimethylated internal standard are seen at $m/e = 320$ (molecular ion) and $m/e = 135$ (base peak).

Survey of methylating reagents for the phase-transfer methylation of niclosamide

The proton NMR hydrolysis studies revealed that 77, 89, 4 and 12% remained, respectively, of methyl iodide, methyl tosylate, dimethyl sulfate, and methyl mesylate after 2 h of stirring under the adopted methylation conditions.

Methyl iodide proved capable of derivatizing niclosamide and the internal standard in high and comparable yield within 1 h (Table I). The presence of substantial O-monomethylated niclosamide and internal standard was noted at shorter reaction times, indicating that N-methylation is a slower process, as expected, than O-methylation. The volatility of the methyl iodide allows easy removal of excess from the derivatization mixture. The cleanup procedure does not completely remove the $(C_4H_9)_4N^+I^-$ from the sample in the benzene–water partition step, so that an appreciable "reagent" peak remains when the GC–ECD determination is run. The peak seen is due to pyrolysis products of $(C_4H_9)_4N^+I^-$. This peak does not affect the analysis. In cases where the concentration of the analyte in the unknown is small and a more concentrated sample must be injected, a rapid, simple Celite chromatography step may be used to remove the $(C_4H_9)_4N^+I^-$ as outlined in the Experimental section.

The other three methylating agents have drawbacks which make them less suitable for the analysis. Each of the three requires an ethanolamine step to destroy excess reagent. Methyl tosylate methylates rapidly and efficiently (Table I) and hydrolyzes at a slow rate but yields several substantial peaks in the chromatogram emerging prior to the internal standard. These could interfere with the analysis of samples containing low concentrations of niclosamide. Dimethyl sulfate methylates efficiently also (Table I), but its half-life due to hydrolysis is less than 15 min under derivatization conditions. Since niclosamide methylates at an appreciably greater rate in dimethyl sulfate than does the internal standard, a sample matrix which enhances the rate of consumption of dimethyl sulfate would produce high results. The methylating agent would be exhausted before the internal standard was completely methylated. Methyl mesylate hydrolyzes only somewhat less rapidly than dimethyl sulfate and is a substantially poorer methylating agent, as shown in Table I.

Phase-transfer methylation of niclosamide and internal standard using methyl iodide

Methyl iodide possesses significant advantages over other methylating agents tested, and therefore was chosen for final development of the method. Fig. 2 shows a reaction rate study (samples MI-1 to MI-8, Table I) for the phase-transfer alkylation

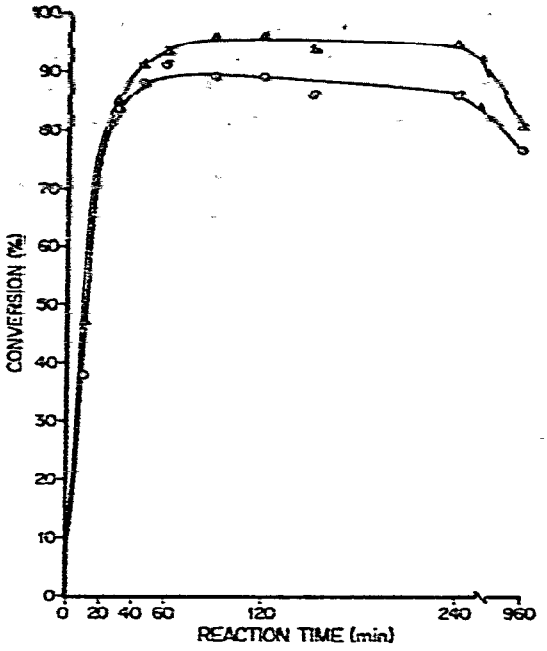

Fig. 2. Derivatization rate study using 50 ml of 3.0% aqueous NaOH, 10 ml of CH_2Cl_2, 2.0 ml of CH_3I and 250 mg of TBACl. △ = niclosamide; ⊙ = internal standard.

method using CH_3I as the alkylating agent. Niclosamide was more rapidly and efficiently methylated than 2′-Cl-4′-NSA, but by $T = 75$ min both reactions leveled off to yield a constant and reproducible conversion ratio. At $T = 75$ min, over 90% of the methyl iodide remained so that the problem encountered with the relatively rapid hydrolysis in the case of dimethyl sulfate was avoided. The absence of O-methyl-2′-chloro-4′-NSA indicates that none of this material remained unreacted in the methylene chloride layer.

An explanation for the lower conversion of the internal standard relative to niclosamide was sought. An experiment was performed to determine if the iodide ion formed by hydrolysis of methyl iodide could tie up, or "poison"[37–39] the phase-transfer cation sufficiently to drastically lower the methylation rate. However, when a blank reaction with added methyl iodide was allowed to stir for 2 h before fortification and subsequent 90-min stirring, conversions of 94.7% for niclosamide and 90.1% for the internal standard resulted. These values are comparable to a normal derivatization run. No appreciable rate reduction occurred as a result of the presence of iodide ion. Therefore, anions derived from niclosamide and the internal standard compete favorably with iodide ion with respect to ion-pair extractability into methylene chloride. This result suggests also that there is no apparent contribution of a $(C_4H_9)_4N^+OH^-$ species in the organic phase to the extractive alkylation process. The presence of a substantial quantity of iodide ion would competitively prevent the ion-pair extraction of hydroxide, and reaction depending on OH^- in the bulk organic phase would be inhibited.

An experiment was run which demonstrated that as little as 25 mg of TBACl

was sufficient to quantitatively extract both the internal standard compound and niclosamide from 50 ml of 3% aqueous base using 10 ml of methylene chloride (see Experimental section). Thus, under the reaction conditions, niclosamide and the internal standard undergo quantitative ion-pair extraction and the aqueous phase serves primarily as a reservoir for hydroxide ion during the alkylation procedure. The enhancement of overall methylation rate due to increased TBACl concentration is attributed to participation of the TBA cation in the reaction occurring in the organic phase.

Linearity studies and applications

The OV-101 stationary phase was chosen to minimize the column temperature required for analysis and to maximize the separation, which depends on the difference of one chlorine between the internal standard and niclosamide. Some early chromatograms utilized Chromosorb W HP as a solid support, but this material was not sufficiently inert to avoid some peak tailing. The use of 5% OV-101 on 100–120 mesh Gas-Chrom Q, a silanized support, provided chromatograms with excellent linearity and minimal tailing.

Standard-curve data for the derivatization of niclosamide using methyl iodide are summarized in Table II. Fig. 3 shows the corresponding GC–ECD traces. The data demonstrate that the linearity of the analytical system is quite good as is the precision at the 25-μg level. The range represented is that corresponding to treatment levels, 0.1 to 1.0 ppm niclosamide in water. Pre-derivatization extraction from aqueous acid and/or post-derivatization cleanup of samples allows the determination of much smaller concentrations if necessary.

TABLE II

STANDARD CURVE DATA FOR NICLOSAMIDE IN WATER USING METHYL IODIDE AS METHYLATING AGENT

Relative standard deviation ($n = 3$) at 25 μg level = 2.1%. Conditions: 50 ml of 3.0% NaOH; 250 mg TBACl; 10 ml CH_2Cl_2; 2.0 ml methyl iodide.

Niclosamide added, x (μg)	*Niclosamide/internal std. peak height ratio, y*	*Niclosamide calculated, x′ (μg)**
0.0	0.0	−0.103
5.122	0.1541	5.02
25.61	0.785	26.0
51.22	1.538	51.0

* Calculated from the least squares line, $y = mx + b$ ($m = 0.03007$; $b = 0.00310$; $r^2 = 0.99987$.

The pre-derivatization extraction cleanup procedure was developed to remove interfering materials from urine and other prospective niclosamide sample matrices which are capable of hydrolyzing the methylating agent. The niclosamide is first extracted from aqueous acid into a diethyl ether–toluene (50:50) layer. This solvent has the advantages of being lighter than water, providing high extraction efficiency, minimizing the tendency for emulsion formation, and yielding the analyte to aqueous base. The aqueous phase is drawn off together with many potential interferants. The niclosamide and internal standard are then extracted into aqueous base, providing a second separation from potential interferants. The phase transfer occurring during the derivatization provides an additional extremely selective separation step.

Determinations were made of Bayluscide purity in technical grade Bayluscide

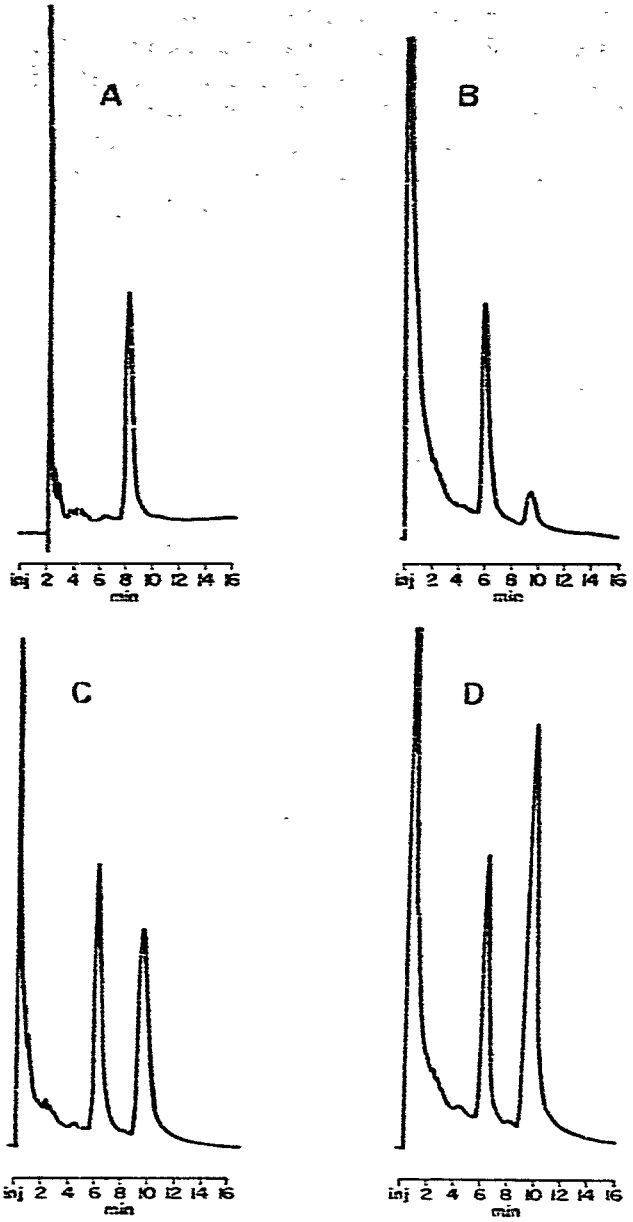

Fig. 3. Representative GC–ECD traces for the derivatization of niclosamide using methyl iodide. Fortifying quantity of niclosamide relative to 25.2 μg of 2′-Cl-4′-NSA internal standard: (A) 0.0 μg; (B) 5.1 μg; (C) 25.6 μg; (D) 51.2 μg. Column 1.83 m × 2 mm I.D. glass packed with 5% OV-101 on 100–120 mesh Gas-Chrom Q. These traces are those corresponding to the standard-curve derivatizations in Table II.

as well as niclosamide in fortified, stagnant water and urine samples. Prederivatization cleanup was used in the latter two cases. The results of these determinations are summarized in Table III. Corresponding representative traces are illustrated in Fig. 4. The relative standard deviation for three determinations is well within 1% for the technical product and for niclosamide in stagnant water. The precision for the urine determination (25-ml urine sample) is about 2%. The values for niclosamide in fortified urine appear to run about 3% low.

The utilization of prederivatization partitioning together with post-derivatization Celite column cleanup of a stagnant water sample fortified with 2.516 μg of internal standard and 0.512 μg of niclosamide demonstrated a minimum detection limit of less than 5 ppb for the method.

The method for niclosamide is applicable, with few modifications, to the analysis of residues in such media of interest as stream and pond sediments and fish. The applicability of the method to determination of the glucuronide conjugate of niclosamide is under investigation.

TABLE III

ANALYSIS OF NICLOSAMIDE IN (A) A TECHNICAL PRODUCT, (B) STAGNANT WATER, AND (C) URINE USING METHYL IODIDE

	Sample designation	*Purity (%) (as ethanolamine salt)*	
A	Chemagro product Accession No. 8749a	95.3 ± 0.53 ($n = 3$)	
Sample matrix	*Niclosamide added (μg)*	*Niclosamide found (μg)*	*Recovery (%)*
B	25.61	25.72 ± 0.08 ($n = 3$)	100.5
C	25.61	24.85 ± 0.53 ($n = 3$)	97.0

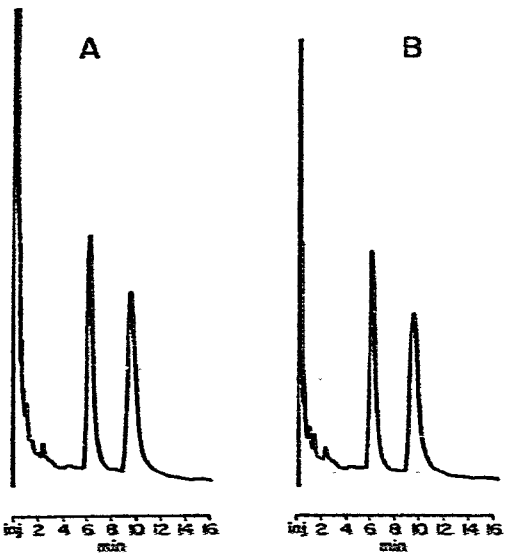

Fig. 4. GC–ECD traces for the determination of niclosamide at the 0.5-ppm level in (A) stagnant water and (B) urine.

The method developed herein for the phase-transfer alkylation (PTA) of niclosamide should be applicable with minimal modification to a number of other pesticides and pesticide metabolites, including salicylanilide and other anilide analogues of niclosamide used as molluscicides and lampricides. Phenols, such as the lampricide 3-trifluoromethyl-4-nitrophenol and the fenitrothion metabolite 3-methyl-4-nitrophenol would be candidates as would certain carbamates, such as N-phenylcarbamates used as herbicides.

The strength of the method lies not only in the potential for efficient yields of chromatographable derivatives, but also in the exclusion of potential interferants during the derivatization process in applications involving demanding sample matrices.

REFERENCES

1 A. F. M. El-Din, *Proc. Int. Conf. Schisto.*, 1 (1978) Inaugural Address.
2 E. A. Malek, *Proc. Int. Conf. Schisto.*, 1 (1978) 359.
3 J. W. Miles, R. T. Taylor, G. C. Robbins and T. B. Blue, *Proc. Int. Conf. Schisto.*, 1 (1978) 469.
4 J. Wellborn, *Progressive Fish-Culturist*, 33 (1971) 32.
5 K. Vermeer, R. W. Risebrough, A. L. Spaans and L. M. Reynolds, *Environ. Pollut.*, 7 (1974) 217.
6 R. P. Rye, Jr. and E. L. King, *Trans. Am. Fish Soc.*, 105 (1976) 332.
7 M. Katz, *Drugs*, 13 (1977) 124.
8 H. Most, *New Eng. J. Med.*, 287 (1972) 495.
9 M. M. Bryden, *Australian Vet. J.*, 45 (1969) 133.
10 J. B. Poole, K. L. Dooley and L. D. Rollins, *J. Amer. Vet. Med. Ass.*, 159 (1971) 78.
11 K. J. Farrington, *Anal. Chem.*, 34 (1962) 1338.
12 R. Strufe, *Bull. World Health Org.*, 25 (1961) 503.
13 R. Strufe, *Pflanzenshutz-Nachrichten (Bayer)*, 18 (1965) 130.
14 N. I. Kokovkin-Shaherbak and S. G. Tiraspolskaya, *Farmatsiya*, 24 (1975) 44.
15 R. F. Sturrock, *Ann. Trop. Med. Parasitol.*, 68 (1974) 427.
16 R. Meredith and G. C. Meredith, *Bull. World Health Organ.*, 46 (1972) 404.
17 E. V. Dehmlow, *Angew. Chem., Int. Ed. Engl.*, 13 (1974) 170.
18 R. A. Jones, *Aldrichemica Acta*, 9 (1976) 35.
19 G. W. Gokel and W. P. Weber, *J. Chem. Educ.*, 55 (1978) 350, 429.
20 A. Brandstrom, *Advances in Physical Organic Chemistry*, 15 (1977) 267.
21 H. D. Durst, M. Milano, E. J. Kitka, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
22 G. Schill, R. Modin, K. Borg and B. A. Persson, in K. Blau and G. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden and Son, Bellmawr, N.J., 1974, pp. 500–529.
23 K. Gustavii, *Acta Pharm. Suecica*, 4 (1967) 233.
24 R. H. Greeley, *J. Chromatogr.*, 88 (1974) 229.
25 J. M. Rosenfeld and J. L. Crocco, *Anal. Chem.*, 50 (1978) 701.
26 O. Gyllenhaal, H. Brötell and P. Hartvig, *J. Chromatogr.*, 129 (1976) 295.
27 M. Ervik and K. Gustavii, *Anal. Chem.*, 46 (1974) 39.
28 B. Lindström and M. Molander, *J. Chromatogr.*, 101 (1974) 219.
29 B. Lindström, M. Molander and M. Groschinsky, *J. Chromatogr.*, 114 (1975) 459.
30 H. Ehrsson and A. Tilly, *Anal. Lett.*, 6 (1973) 197.
31 J. A. F. De Silva and I. Bekersky, *J. Chromatogr.*, 99 (1974) 447.
32 V. E. Schraufstatter, W. Meiser and R. Gonnert, *Z. Naturforsch. B.*, 16 (1961) 95.
33 S. Cacchi, F. LaTorre and D. Misiti, *Synthesis*, (1977) 302.
34 A. McKillop, J. C. Fiaud and R. P. Hug, *Tetrahedron*, 30 (1974) 1379.
35 R. Brehme, *Synthesis*, (1976) 113.
36 F. C. Churchill, *Dissertation*, University of Georgia, Athens, Ga., 1979.
37 J. E. Gordon and R. E. Kutina, *J. Amer. Chem. Soc.*, 99 (1977) 3903.
38 C. M. Starks and R. M. Owens, *J. Amer. Chem. Soc.*, 95 (1973) 3613.
39 A. W. Herriott and D. Picker, *J. Amer. Chem. Soc.*, 97 (1975) 2345.