Journal of Chromatography, 307 (1984) 420—425
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2045

Note

Determination of germicidal phenols in blood by capillary column gas chromatography

RICHARD E. CLINE*, LOUISE W. YERT and LARRY L. NEEDHAM

Center for Environmental Health, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received September 19th, 1983; revised manuscript received December 15th, 1983)

The investigation of two hyperbilirubinemia epidemics that affected infants in hospital nurseries suggested a causal association with the excessive use of a disinfectant containing *p-tert.*-amylphenol (3%), *o*-phenylphenol (0.5%), and *o*-benzyl-*p*-chlorophenol (6.4%). In one investigation, serum phenols were determined by gas chromatography (GC) with flame ionization detection, which gave a detection limit of 1 ppm in serum [1]. In the other, serum phenols were determined by high-performance liquid chromatography with dual ultraviolet detectors and a fluorescent detector [2]. Although the latter was more sensitive, quantification was difficult because of interferences in some samples with peaks of *p-tert.*-amylphenol and *o*-benzyl-*p*-chlorophenol.

The present method of determining germicidal phenols in serum is somewhat similar to our published method for determining pentachlorophenol [3]. The germicidal phenols differ from pentachlorophenol in that they are much less sensitive to electron-capture detection and also less lipophilic. Consequently, the present procedure differs mainly in that phenols are derivatized with an electrophoric reagent, and a more polar extraction solvent is used.

To enhance the electron-capture response of various phenols, Lamparski and Nestrick [4] derivatized them with heptafluorobutyrylimidazole and analyzed extracts by GC on a packed column. McKague [5] derivatized phenols with heptafluorobutyric anhydride and used a capillary column.

Some previous bioanalytical examples of the use of electron-capturing reagents for derivatizing phenolic groups can be cited. Trifluoroacetic anhydride was used in methods for diethylstilbestrol [6] and dopamine [7] in urine, and morphine [8] in serum. Pentafluoropropionic anhydride was reacted with

0378-4347/84/$03.00

catecholamines [9] and pentafluorobenzoyl chloride with phenolic acids [10] in urine analyses.

EXPERIMENTAL

*Reagents**

Heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL, U.S.A.), Dowicide 1 (*o*-phenylphenol) from Dow Chemical (Midland, MI, U.S.A.), Pentaphen No. 67 (*p-tert.*-amylphenol) from Pennsalt (Philadelphia, PA, U.S.A.), Santophen 1 (*o*-benzyl-*p*-chlorophenol) from Monsanto (St. Louis, MO, U.S.A.), and *p*-phenylphenol from Chem Service (West Chester, PA, U.S.A.). Isooctane and diethyl ether were of distilled-in-glass quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Pyridine was supplied by Mallinckrodt (St. Louis, MO, U.S.A.). A lecture bottle of trimethylamine was obtained from Matheson Gas Products (Morrow, GA, U.S.A.). Buffer of pH 9.2 was prepared by diluting 1.236 g of boric acid and 53.4 ml of 0.2 *M* sodium hydroxide with water to 200 ml. Buffer of pH 8 was prepared by diluting 1.236 g of boric acid and 7.9 ml of 0.2 *M* sodium hydroxide with water to 200 ml.

Synthesis of heptafluorobutyrates of phenols

To 5 ml of diethyl ether containing 1.23 g (7.2 mmol) of *p*-phenylphenol were added 2 ml of heptafluorobutyric anhydride (8 mmol). Trimethylamine was bubbled into the mixture for a few minutes, after which it was allowed to stand 1 h at room temperature in a tube with a polytrifluoroethylene (PTFE)-lined cap. More trimethylamine was added, and 15 min later the mixture was washed three times with 10 ml of a pH 6 buffer and once with a pH 8 buffer. The organic layer was evaporated to a low volume under a stream of nitrogen. A product weighing 1.13 g was removed by filtration and recrystallized several times from isooctane. The *p*-phenylphenol heptafluorobutyrate melted at 72—73° C.

Similar procedures were used to prepare heptafluorobutyrates of the other three phenols. These derivatives were purified by distillation at reduced pressure to give boiling points at pressures (mmHg) as follows: *o*-benzyl-*p*-chlorophenol derivative, 85° C (0.05 mmHg); *o*-phenylphenol derivative, 68° C (0.15 mmHg); and *p-tert.*-amylphenol derivative, 53° C (0.10 mmHg).

Gas chromatography—mass spectrometry of phenol heptafluorobutyrates

The instrument used was a Finnigan 4000 equipped with a quadrupole spectrometer and a 1.8 m × 2 mm I.D. GC column packed with 3% OV-1. All four phenol derivatives showed only one GC peak. Details of the mass spectra are shown in Table I.

Extraction and derivatization

Each 0.5-ml sample of blood serum was spiked with 4 μl of an acetone

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

MASS SPECTRA OF PHENOL HEPTAFLUOROBUTYRATES

It is noteworthy that the spectrum of the *o*-phenylphenol derivative has a large peak at *m/z* 197 (from loss of C_3F_7) which is not in the spectrum of the *p*-phenylphenol derivative.

Phenolic compound	*m/z* (percent relative abundance)
o-Benzyl-*p*-chlorophenol	414[*M*⁺](21), 217(100), 201(17), 181(19), 165(57), 152(32)
o-Phenylphenol	366[*M*⁺](50), 169(100), 197(83), 153(25), 141(56), 115(50)
p-Phenylphenol	366[*M*⁺](63), 169(100), 153(11), 141(51), 115(36), 69(17)
p-tert.-Amylphenol	360[*M*⁺](3), 331(100), 303(23), 275(6), 169(10), 115(13)

solution of *p*-phenylphenol (internal standard). Calibration samples were spiked with acetone containing three germicidal phenols and internal standard. Spiked samples were rotated slowly for 1 h in tubes fitted with PTFE-lined screw caps. To each tube was added 0.5 ml of 2 *M* sulfuric acid, followed by 3 ml of 20% diethyl ether in isooctane. In order to decrease emulsification, the tube was rotated slowly for 2 h. Following centrifugation, 2 ml of each top layer was transferred to another tube and derivatized by adding successively 50 µl of pyridine and 50 µl of heptafluorobutyric anhydride, shaking for 1 min, and allowing the mixture to stand for 20 min at room temperature. By-products were removed by washing the extracts (shaking 30 sec each time) with 4 ml of pH 9.2 borate buffer followed by 4 ml of pH 8 borate buffer. The washed extracts were dried with anhydrous sodium sulfate before they were analyzed by GC.

Gas chromatography

The Tracor 560 gas chromatograph (Tracor, Austin, TX, U.S.A.) was equipped with a ^{63}Ni electron-capture detector and linearizer (constant-current pulsed mode). It was fitted with an 11 m × 0.25 mm I.D. fused-silica capillary column with methyl silicone coating (WCOT) obtained from Hewlett-Packard (Avondale, PA, U.S.A.). With a Varian 8000 autosampler, 1.5 µl was injected in the splitless mode for 1 min at 58°C, after which the temperature was programmed up to 178°C at a rate of 3°C/min, the last peak of interest emerging at 126°C. The detector temperature was 350°C. The injection port temperature was normally kept at 225°C but increased to 265°C during the 1-min injection period. The carrier gas was hydrogen at a flow-rate of 2.4 ml/min (linear velocity of 69 cm/sec with methylene chloride). The make-up gas was argon—methane at a flow-rate of 50 ml/min. The purge flow-rate from the injection port was 50 ml/min. Peak areas were quantified by means of the Supergrator-3 (Columbia Scientific Industries, Austin, TX, U.S.A.) placed between the detector and the recorder.

Quantitative analysis

Standard curves for each germicidal phenol were obtained by the method of standard addition with *p*-phenylphenol used as internal standard. The ratios of peak areas (germicidal phenol/internal standard) were plotted as a function of increasing concentrations of germicidal phenol in serum. The normal standard curves were obtained from analyses of eight samples spiked with

p-tert.-amylphenol at levels of 0—554 ng/ml, with *o*-phenylphenol at 0—405 ng/ml and *o*-benzyl-*p*-chlorophenol at 0—642 ng/ml. All samples were spiked with internal standard at 394 ng/ml. The average of correlation coefficients for standard curves from three experiments was 0.9979 ± 0.08% (relative standard deviation).

RESULTS AND DISCUSSION

Heptafluorobutyric anhydride (HFBA), pentafluorobenzoyl chloride, pentafluorobenzyl bromide, and 1-fluoro-2,4-dinitrobenzene have been tested as derivatizing agents in order to increase the electron-capture response. These agents have been used by McCallum and Armstrong [11] to prepare thymol derivatives and determine their relative sensitivity to electron-capture detection. The pentafluorobenzoate was found to be the most sensitive, being

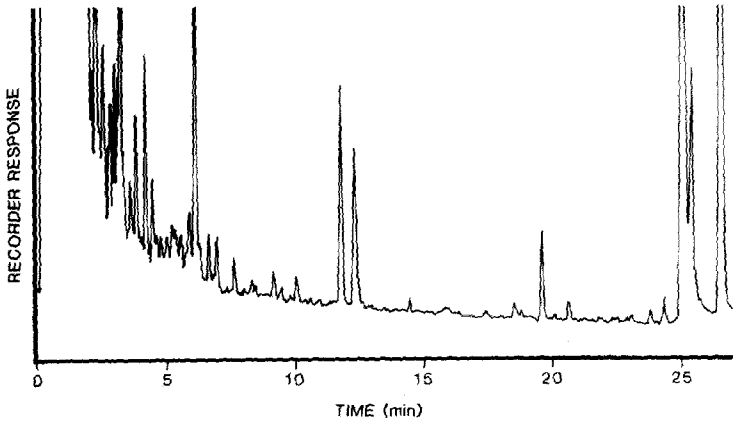

Fig. 1. Chromatogram of blank serum extract treated with HFBA. GC on capillary column as described in Experimental.

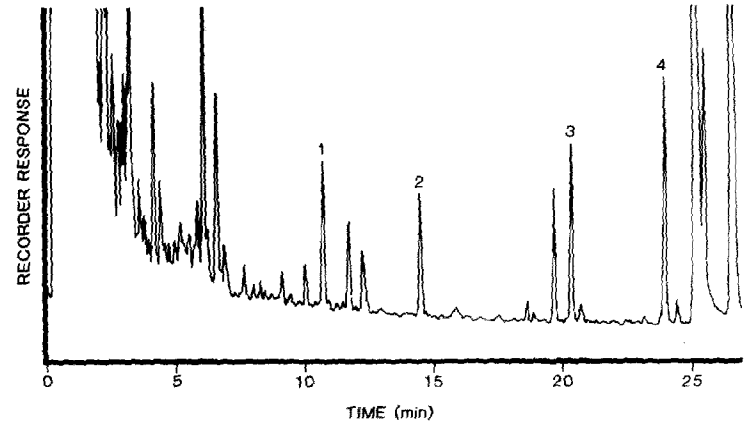

Fig. 2. Chromatogram of germicidal phenol-supplemented serum extract treated with HFBA. Heptafluorobutyrate peaks: 1 = *p-tert.*-amylphenol (101 ng/ml); 2 = *o*-phenylphenol (73 ng/ml); 3 = *p*-phenylphenol (141 ng/ml); 4 = *o*-benzyl-*p*-chlorophenol (102 ng/ml). GC conditions as described in Experimental.

seven times more potent than the heptafluorobutyrate, the second most sensitive. In our procedure, heptafluorobutyric anhydride appeared to be the most suitable reagent from the standpoint of percentage of recovery, nature of by-products, and volatility of derivatives.

The reaction of HFBA with an extract of serum unsupplemented with germicidal phenols produced a large number of electron-capturing compounds, as shown in Fig. 1. Most of these very early peaks were also found in 20% diethyl ether in isooctane reacted with HFBA and, therefore, result mainly from reagent impurities and/or reaction by-products. Fig. 2 shows a chromatogram of an extract of plasma spiked with germicidal phenols. A comparison of these two chromatograms shows that interferences with the germicidal phenols are minimal. However, a small amount of interference is present at the retention time of the HFBA derivative of *o*-benzyl-*p*-chlorophenol. Much higher interferences occurred in early experiments in which a column packed with 5% SP 2100 was used. The capillary column clearly offers advantages over packed columns in separating all the components of these complex mixtures.

Peak heights varied considerably for some of the unknown constituents of samples of the same serum pool, for example, the two peaks with retention times near 12 min in Figs. 1 and 2. The explanation may be that such constituents form unstable derivatives with HFBA. However, effects of reaction conditions have not been investigated.

Extraction solvents other than 20% diethyl ether were tried. Isooctane has a boiling point which makes it ideal for use in splitless injection on a capillary column, but it has less than the nearly 100% extraction efficiency of 20% diethyl ether in isooctane. Benzene or 50% diethyl ether in isooctane extracted much larger amounts of substances which interfered with peaks of interest.

Results of quantitative analyses in which we used the internal standard and calibration curves are shown in Table II. Each 0.5-ml sample of serum was spiked with 4 μl of acetone containing internal standard and 4 μl of acetone containing a mixture of three germicidal pehnols. Table II shows the amounts

TABLE II

RECOVERY OF GERMICIDAL PHENOLS FROM SERUM

Experiment	*p-tert.*-Amylphenol		*o*-Phenylphenol		*o*-Benzyl-*p*-chlorophenol	
	Added (ng/ml)	Recovery (%)	Added (ng/ml)	Recovery (%)	Added (ng/ml)	Recovery (%)
1	203	92	148	95	235	91
2	203	101	148	109	235	102
	406	100	299	101	470	105
3	51	94	37	135	59	103
	102	98	74	126	118	96
	203	86	148	106	235	88
	406	106	297	107	470	97
	610	82	446	98	706	71

of phenols added to the serum in ng/ml and the percentage of these phenols found by the analytical procedure (percent recovery). With a few exceptions, the recoveries ranged from 86 to 109%. The practical limit of detection for each phenol in serum appears to be near 20 ng/ml.

REFERENCES

1 D.K. Wysowski, J.W. Flynt, M. Goldfield, R. Altman and A.T. Davis, Pediatrics, 61 (1978) 165.
2 L.L. Needham, R.H. Hill, D.C. Paschal and S.L. Sirmans, Clin. Chim. Acta, 107 (1980) 261.
3 L.L. Needham, R.E. Cline, S.L. Head and J.A. Liddle, J. Anal. Toxicol., 5 (1981) 283.
4 L.L. Lamparski and T.J. Nestrick, J. Chromatogr., 156 (1978) 143.
5 A.B. McKague, J. Chromatogr., 208 (1981) 287.
6 A.E. Tirpenou, S.D. Kilikidis and A.P. Kamarianos, J. Ass. Offic. Anal. Chem., 66 (1983) 1230.
7 D.D. Clarke, S. Wilk, S.E. Gitlow and M.J. Franklin, J. Gas Chromatogr., 5 (1967) 307.
8 J.E. Wallace and H.E. Hamilton, Anal. Chem., 46 (1974) 2167.
9 K.P. Wong, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 47 (1973) 215.
10 M. Makita, S. Yamamoto, K. Ono and T. Katagi, J. Chromatogr., 166 (1978) 294.
11 N.K. McCallum and R.J. Armstrong, J. Chromatogr., 78 (1973) 303.