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ELECTRON-CAPTURE, CAPILLARY COLUMN GAS CHROMATOGRAPHIC DETERMINATION OF LOW-MOLECULAR-WEIGHT DIOLS IN SERUM

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SUMMARY

Research on alcoholism has revealed that concentrations of 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol may be greater in the serum of chronic alcoholics than in the serum of social drinkers and nondrinkers. In connection with one of these studies, we developed methodology to determine these diols at the micromolar levels in 500 serum samples. The procedure consisted primarily of extraction of the serum with acetonitrile containing internal standard. The extract was then concentrated to dryness and reacted with *p*-bromophenylboric acid. The reaction mixture was injected into a gas chromatograph fitted with a capillary column and an electron-capture detector. The total coefficients of variation were best for 1,2-propanediol, 6.82 and 10.00%, and worst for *d,l*-2,3-butanediol, 13.64 and 19.22%. The observed means for the analytes were all within 10% of the spiked level.

INTRODUCTION

In recent years research on alcoholism has revealed that butanediol has been associated with alcoholism [1—3]. Preliminary work at the National Institute on Alcohol Abuse and Alcoholism (NIAAA) indicated that three diols, 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol, may be associated with alcoholism. A joint study among the Centers for Disease Control (CDC), Harvard University's School of Medicine and NIAAA was designed to answer several questions regarding the concentrations of these low-molecular-weight diols in the blood serum of different populations. We developed a method to determine 1,2-propanediol, *d,l*-2,3-butanediol, and *meso*-2,3-butanediol at the micromolar levels in 500 serum samples and appropriate quality control samples. In trace analysis, procedures can be divided into several steps, which often include extraction, chromatography and detection. Previous gas chromatographic (GC) procedures that were developed at NIAAA for determining these diols included either direct injection of deproteinizated serum or injection of a methyl ethyl ketone extract. According to the authors, these procedures

suffered from inadequate sensitivity and extraction reproducibility. Although most reported procedures for ethylene glycol have similar deficiencies [4—6], a recent article for determining ethylene glycol [7] in blood served as a basis for our method. To determine the diols in which we are interested, we made many changes in the basic method. For example, we used a different extraction solvent and, as the derivatizing agent, a different boronic acid. We also added an internal standard and used capillary column chromatography with electron-capture detection. These changes made the analysis faster and more precise, sensitive and specific.

When our study was nearly completed, we learned that a procedure [8] similar to ours for determining ethylene glycol in serum had been submitted for publication. Our method, however, differed in several aspects: it determined different analytes; a different internal standard and a different derivatizing agent were used; and capillary column chromatography, with electron-capture detection, was employed.

EXPERIMENTAL*

Chemicals and reagents

The following were used: *p*-bromophenylboric acid and 1,2-butanediol (Aldrich, Milwaukee, WI, U.S.A.); propylene glycol (USP), ethyl acetate (spectranalyzed) and acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.); *d,l*-2,3-butanediol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and 1,3-propanediol (Chem Service, West Chester, PA, U.S.A.). *meso*-2,3-Butanediol was fractionated from a 1:1 mixture of *meso* and racemic 2,3-butanediols (K & K Labs., Plainview, NY, U.S.A.) by aqueous liquid chromatography [9]. This mixture of 2,3-butanediols was used to fortify our quality control pools. The propylene glycol was further purified by distillation, boiling point 189°C.

Apparatus

A Hewlett-Packard Model 5713A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.), equipped with a constant-current ^{63}Ni electron-capture detector, a Varian Model 8000 autosampler (Varian, Walnut Creek, CA, U.S.A.) and a Hewlett-Packard 3390A integrator recorder were used. Instrument operating temperatures were as follows: detector, 350°C; injector, 200°C; column oven, 120°C. The carrier gas was helium (99.999 UHP), at a flow-rate of 1 ml/min; the detector make-up gas was argon—methane (95:5), at a flow-rate of 47 ml/min. The system was operated in a fully automated mode. Split mode of injection was incorporated with a split ratio of 1:150; the inlet splitter was packed with silanized glass wool. A 12-m fused-silica capillary column coated with methyl silicone fluid (Hewlett-Packard), was used. The injection volume was 1 μl.

Procedure

One milliliter of serum and 6 ml of acetonitrile, which contained the internal

*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.

standard (1,2-butanediol) at a concentration of 83 μM, were volumetrically pipetted into a 15-ml round-bottom tube. The tube was fitted with a PTFE-lined screw cap, vortexed for 3 min, and then centrifuged at 2400 g for 30 min. The extract was decanted to another 15-ml round-bottom tube. This tube was placed in a 70°C water bath, and the extract was concentrated to a reduced volume (ca. 0.2 ml) by using a stream of nitrogen. The nitrogen passed through a multiport manifold fitted with 18 needles, each of which was directed into a tube. Each extract was then reduced to dryness by passing a gentle stream of nitrogen through a 9-in. Pasteur pipet into the tube, which was hand-held at room temperature and continually rotated to disperse the residue.

One milliliter of a 1% *p*-bromophenylboric acid in ethyl acetate solution was added to each tube. The mixture was vortexed and allowed to stand at room temperature for a minimum of 30 min. The reaction mixture was again vortexed and then pipetted into automatic sampler vials. Samples of 1 μl were injected by the autosampler. Vials containing acetone were placed after each sample, and the automatic sampler was washed with acetone after each sample injection. Including the wash cycle and data reporting time, the analysis time was approx. 20 min.

Quantitation

Bovine sera spiked at six different concentrations, ranging from 20 to 1000 μM with each of the three analytes, were analyzed with each run. These standards were injected at both the beginning and the end of the gas chromatographic sequences. The areas for all three analytes and the internal standard were entered into a computer. Peak area ratios (analyte/internal standard) for each of the analytes in the standards were calculated and plotted on a log/log scale versus the concentration of the analyte in the corresponding standard. The respective calibration curves were constructed, and the best fitting line was found by using a least-squares regression method. The concentration of each of the analytes in the unknowns was computed.

Quality assurance

To estimate the precision and accuracy of our analyses, we prepared and analyzed quality control samples. The more concentrated quality control samples (pool 3) were prepared by spiking human serum with the analytes of interest in water; this more concentrated pool was then diluted with more serum to prepare pool 2. Pool 1 was the serum with no analytes added. The pools were each stirred overnight at 4°C and then filtered under sterile conditions. The filtrate was dispersed in 1-ml aliquots into vials and frozen. With each analytical run, which consisted of 20 unknown samples and 6 standards, 2 quality control samples from pools 2 and 3 were also analyzed.

RESULTS AND DISCUSSION

Procedure

The interaction of diols with traditional GC packing materials leads to poor separation and peak symmetry, especially in the less concentrated samples, and the end result is nonlinearity of response. Therefore, the analytes must be derivatized.

Several criteria for selecting the appropriate diol must be met. The derivative should not interact with the contents of the column, and it should be of sufficient molecular weight to be retained on the column. On the other hand, it must be capable of resolving the very similar butanediols and propanediol. Another factor in selecting the derivatizing agent is that it should not be monofunctional, which might lead to mixed mono- and di-derivatives. *p*-Bromophenylboric acid seemed to be an ideal candidate for meeting these criteria. The reaction with the diols of interest is shown in Fig. 1. In addition, the *p*-

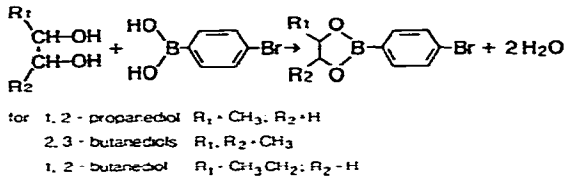

Fig. 1. Reaction of the diols with *p*-bromophenylboric acid to form the cyclic *p*-bromophenylboronates.

bromophenylboronates exhibit increased stability and electron-capture detector sensitivity relative to the other boronates [10]. The use of electron-capture detection, as compared with flame ionization detection, permits greater sensitivity and selectivity. We used a detector temperature of 350°C because higher detector temperatures result in less detector contamination and because the dissociative capture mechanism which the boronates undergo [11] is more efficient at higher temperatures. The only disadvantage in using the boronates is that the derivatization of *meso*-2,3-butanediol might have slower kinetics than the *d,l*-form. The reason is that in the preferred conformation of the butanediols (that is, with the methyl groups *anti* to each other), the *meso* form also has its hydroxyl groups *anti* to each other, whereas the *d,l*-form has its hydroxyl groups *gauche* to each other (Fig. 2); this *gauche* conformation is

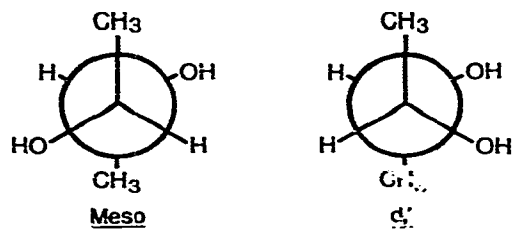

Fig. 2. The *anti* conformation of *meso*- and *d,l*-2,3-butanediol.

ideal for the formation of the bridged boronates. Although we did not study the kinetics, we saw no significant difference in the recoveries of the *meso*- compared with the *d,l*-2,3-butanediol derivative. Determining the percent recovery of each separate step was difficult because the diols apparently react with derivatizing agent that remains from previous injections on the head of the column or in the injector. This was shown by injecting the diols themselves into the

system after derivatized samples had been analyzed. The corresponding derivatized diols were then detected by electron capture and mass spectrometry. This on-column method and other methods for forming the boronates from diols were recently reported [12].

The electron-impact fragmentation patterns of these bromophenylboronate derivatives of the analytes of interest are very similar to the mass spectra of the corresponding phenylboronate derivative [13, 14]. As shown in Fig. 3, the molecular ion at *m/z* 254 for the *d,l*-2,3-butanediol derivative is pronounced. Furthermore, corresponding peaks are present at *m/z* 256 (primarily because of ^{81}Br) and at 253 and 255 (primarily because of ^{10}B). The base peak at *m/z* 239 is due to the loss of a methyl group. Other ions of prominent size are at *m/z* 182 (C_6H_4BBrO), 183 (C_6H_5BBrO), 103 (C_6H_4BOO), and 77 (C_6H_5).

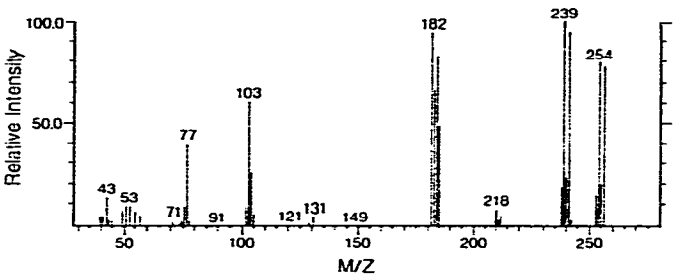

Fig. 3. The 70-eV electron-impact mass spectrum of the *p*-bromophenylboronate of *d,l*-2,3-butanediol.

Once we decided to use the boronate derivatives, the next problem was to free these water-soluble diols from serum. Extraction with semi-polar, water-immiscible solvents, such as methyl ethyl ketone and ethyl acetate, resulted in low and sporadic recoveries. We then used acetonitrile as both a deproteinizing and extracting solvent; the water was removed by using heat and a gentle stream of nitrogen. The volume of 6 ml of acetonitrile to 1 ml of water was used because these solvents form a 5.1:1 azeotrope which boils at 76.5°C [12]. This allows water to be rapidly removed at a lower temperature than would otherwise be possible. Nonetheless, this evaporation step could be one of the most error-prone steps in the analyses. For example, when the diols were spiked into water and concentrated, recovery was low. However, when the analytes were spiked into serum and concentrated to a low volume and then reduced to dryness by the hand-held procedure, the recoveries were good. Apparently, the serum lipids entrap the diols and prevent their loss. We were concerned about possible losses in this concentration step, and a primary reason for selecting 1,2-butanediol as the internal standard was that its reported boiling point (192—194°C) is close to those of the analytes of interest [14]. Another reason for selecting 1,2-butanediol is that it forms a five-membered cyclic boronate, as do the analytes of interest (Fig. 1); other diols, such as 1,3-propanediol, form a six-membered boronate and they reportedly have different stability properties from the five- and seven-membered rings [11]. 1,3-Propanediol was also not selected as the internal standard because it was found in the undistilled alcoholic beverages that we analyzed.

Precision and accuracy

We estimated precision and accuracy in our 26 runs of unknown samples by analyzing pools 2 and 3 in duplicate in each run. A gas chromatogram of pool 2 is shown in Fig. 4. The same derivatives were also separated at 120°C on a 1.83 m × 4 mm I.D. glass column packed with 3% SE-30 on 80—100 mesh Supelcoport; however, the packed-column separation resulted in less resolution and increased analysis time.

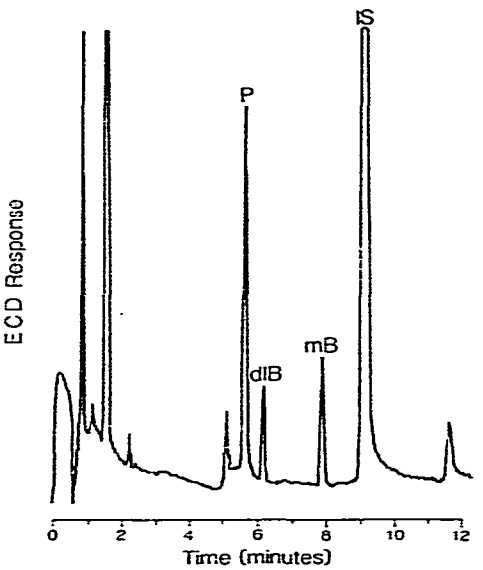

Fig. 4. Gas chromatogram of the *p*-bromophenylboronates of 1,2-propanediol (P); *d,l*-2,3-butanediol (dlB); *meso*-2,3-butanediol (mB); and 1,2-butanediol (IS) in Pool 2. Conditions as described in text.

As shown in Table I, the observed means for the analytes in the quality control pools in all instances were within 10% of the spiked levels as depicted by the percent bias. These results are not corrected for the very small amount of 1,2-propanediol found in the base pool. As expected, the coefficients of variation of the analysis for each analyte were lower for the more concentrated pool. Quality control charts for each analyte in each pool were generated by plotting the values for each run. The chart for *d,l*-2,3-butanediol in pool 2 is

TABLE I

ESTIMATE OF PRECISION AND ACCURACY OF THE METHOD

Analyte	Pool	Spiking level (μM)	Mean determined (μM)	Bias (μM)	Bias (%)	Standard deviation (μM)	C.V. (%)	95% Control limits (μM)	N
1,2-Propanediol	2	50	55	+ 5	+10.0	5.5	10	65.8/44.2	51
1,2-Propanediol	3	700	693	− 7	− 1.0	47.2	6.8	786/600	52
d,l-2,3-Butanediol	2	25	27	+ 2	+ 8.0	5.1	18.9	37.0/17.0	52
d,l-2,3-Butanediol	3	350	385	+35	+10.0	52.6	13.7	488/282	52
meso-2,3-Butanediol	2	25	24	− 1	− 4.0	3.4	14.2	30.7/17.3	52
meso-2,3-Butanediol	3	350	339	−11	− 3.1	32.6	9.6	403/275	52

shown in Fig. 5. The 95% and 99% control limits, which were used to determine if the method was in control, are calculated by subtracting from and adding to the mean the standard deviation times 1.96 and 2.58, respectively. In all of the analyses, we experienced the greatest deviation from the mean in the earlier runs. This deviation was decreased by lowering the column temperature.

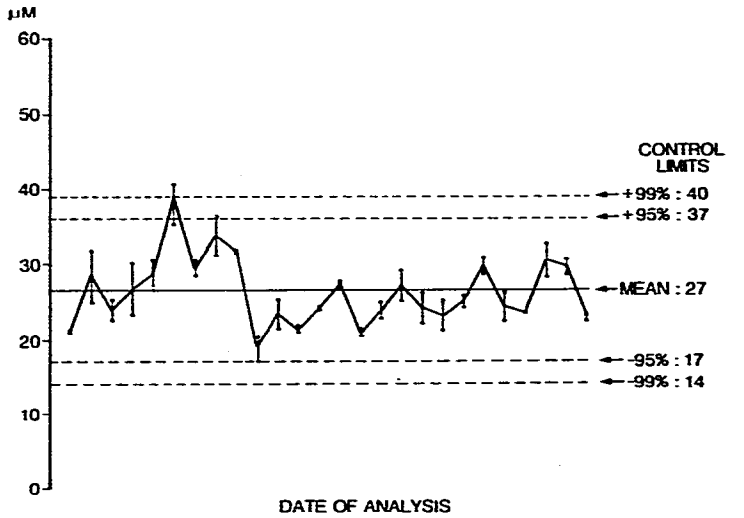

Fig. 5. Quality control chart for the observed values of *d,l*-2,3-butanediol in pool 2.

Recovery

Because our supply of the derivatizing agent was limited, we prepared and purified only the *p*-phenylboronate of *d,l*-2,3-butanediol. When this diol was added to serum at a concentration of 700 μM, the recovery was approximately 87%.

Linearity and reproducibility of standard curves

Log/log plots were used because of the large concentration range (20.0—1000 μM). The linearity of the standard curves for the 26 runs was very reproducible as evidenced by the mean correlation coefficients (± standard deviation) of 0.9968 (±0.0030), 0.9950 (±0.0044) and 0.9960 (±0.0044) for 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol, respectively. The reproducibility of the standard curves was quite precise as indicated in Table II for 1,2-propanediol and *d,l*-2,3-butanediol. We calculated these values from the linear regression line using the area ratios for each point on the curve. These points represent 26 standard curves taken from each run over a one-month period.

Quantitation limit

Various definitions [15, 16] for the term "limit of quantiation" or "limit of determination" have been discussed. In this study, we spiked two serum pools with each analyte at 5.0 μM and at 10.0 μM. We took 19 aliquots from each

TABLE II

MEAN CONCENTRATIONS CALCULATED FOR STANDARDS FROM LINEAR REGRESSION LINES

Number of standard curves is 26 runs over a one-month period.

Target standard value	1,2-Propanediol* values found	*d,l*-2,3-Butanediol* values found
20	20 ± 2	21 ± 2
50	50 ± 3	50 ± 4
100	100 ± 10	99 ± 12
500	480 ± 20	490 ± 30
1000	1000 ± 110	1020 ± 70

*Mean ± standard deviation in μM.

pool and, in a separate analytical run, one analyst analyzed them under the same conditions that had been used for the unknowns. As shown in Table III, the accuracy, as reflected by percent bias, and the standard deviation were similar for either concentration. In this determination, the percent differences for the lower concentrations are not as clinically significant as for the higher concentrations. Nonetheless, because of the increasing relative standard deviation with lower concentrations, our lower limit of quantitation was set at 5.0 μM.

TABLE III

ESTIMATE OF PRECISION AND ACCURACY FOR THE METHOD AT LOW CONCENTRATIONS

Analyte	Spiked level (μM)	Observed mean	Bias (%)	Within-run standard deviation	C.V. (%)
1,2-Propanediol	10	12	20.00	1.0	8.33
1,2-Propanediol	5	5.5	10.00	1.5	27.27
d,l-2,3-Butanediol	10	9.5	− 5.00	1.6	16.84
d,l-2,3-Butanediol	5	4.6	− 8.00	2.0	43.48
meso-2,3-Butanediol	10	9.1	− 9.00	2.4	26.37
meso-2,3-Butanediol	5	4.4	−12.00	1.2	27.27

Application

The method described herein has been used for determining serum levels of the three analytes in the 500 samples prevously mentioned. Results of this joint study by the Harvard School of Medicine, the National Institute for Alcohol Abuse and Alcoholism, and the Centers for Disease Control will be reported soon.

This method could also be used for determining other diols and other difunctional compounds of clinical and toxicological interest.

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