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Short communication

Serum ethylene glycol by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography procedure for detection and quantitation of ethylene glycol in serum is described. Ethylene glycol and internal standard are derivatized with benzoyl chloride under alkaline conditions, purified by solid-phase extraction and analyzed by HPLC with UV detection. Analytical recovery of ethylene glycol ranges between 96 and 103%. The calibration curve is linear from 20 to 2000 mg/l. The limits of detection and quantitation are 10 and 20 mg/l, respectively. Assay imprecision is 4.8% or less. The assay is free from common interferences and provides increased sensitivity, improved precision and extended linearity.

Keywords: Ethylene glycol

1. Introduction

Rapid analytical confirmation of ethylene glycol poisoning is important in defining appropriate clinical management. Numerous methods are available for detection and/or quantitation of ethylene glycol. Enzymatic oxidation using alcohol dehydrogenase [1], glycerol dehydrogenase [2,3] and chemical oxidation to formaldehyde with a subsequent color reaction [4] provide rapid results. However, these oxidation assays are subject to interference from glycerol, propylene glycol and other chemicals. Accordingly, positive results need to be confirmed by a more definitive method. Gas chromatography, with or without on-column derivatization, is rapid and more selective and thus may be used alone or as a confirmation method [5–7]. However, surface adsorption of ethylene glycol can complicate gas chromatographic analysis by producing peak tailing and/or carryover from one injection to the next [7]. As an alternative to gas chromatography, we evaluated a previously described assay using high-performance liquid chromatography (HPLC) following benzoyl chloride derivatization [8]. This method, although more time consuming than gas chromatography, can be used alone or as a confirmation method. We report numerous modifications which enhance the original HPLC assay and provide for more rapid analysis.

2. Experimental

2.1. Materials and reagents

Ethylene glycol, 1,3-propanediol, glycine and benzoyl chloride were purchased from Sigma (St.

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Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Curtin Matheson Scientific (Houston, TX, USA). Bovine serum was purchased from Pel-Freez (Rogers, AR, USA).

2.2. Standards and controls

Ethylene glycol stock standard and control were prepared in water. Working standards and controls were prepared from stock solutions in bovine serum. Internal standard, 500 mg/l 1,3-propanediol [9], was prepared in water. Stock and working standards and controls were stored at −70°C.

2.3. Derivatization

A 100-μl volume of each standard, control and patient sample was mixed with 100 μl of internal standard and 500 μl of 8 *M* NaOH. A 50-μl volume of benzoyl chloride was added to each specimen with immediate vortex-mixing. The mixture was incubated at ambient temperature for 5 min. The reaction was terminated by addition of and mixing with 30 μl of 1% glycine.

2.4. Extraction

Each specimen mixture was applied to an Analytichem Chem Elut CE1001 diatomaceous earth column (Harbor City, CA, USA). After 5 min, 5 ml of pentane was added to each column. The benzoyl derivatives of ethylene glycol, the internal standard and other alcohols and glycols were extracted into the eluate. This step was repeated giving a total volume of approximately 10 ml. The pooled eluate was evaporated at 46°C under a stream of air. The residue was resuspended with 400 μl of methanol and 10 μl were injected onto the HPLC system.

2.5. HPLC apparatus and conditions

The HPLC system consisted of a Beckman Model 114M pump, Model 504 autoinjector and Model 164 UV–Vis detector (Brea, CA, USA). The system was outfitted with a 4 μm Waters Nova Pak C_{18} column, 15 cm×3.9 mm I.D. (Milford, MA, USA). The column was maintained at ambient temperature. The mobile phase was acetonitrile–water (50:50, v/v). The flow-rate was 2.5 ml/min. The column eluate was monitored at 237 nm (0.02 absorbance units full scale). Chromatograms were recorded using an Hewlett-Packard HP 3396A integrator (Avondale, PA, USA).

2.6. Assay validation

The calibration curve ranging from 20 to 2000 mg/l was constructed by least-squares regression of the ratios of ethylene glycol peak height to internal standard peak height plotted versus ethylene glycol concentration. The limit of detection was defined as the concentration that produced at least three times the background signal produced by analysis of ethylene glycol-free serum. The limit of quantitation was defined as the lowest ethylene glycol concentration tested for which the lower extent of the 95% confidence interval from the calibration curve did not intersect the *x*-axis. Selectivity was based on retention time and relative retention time. The latter was calculated by dividing the retention time for ethylene glycol by the retention time for the internal standard and comparing the result to the same calculation derived from standards. Within-run imprecision was determined by repeated ($n=10$) analysis of high and low concentration controls in a single analytical run. Between-run imprecision was based on repeated analysis of the same high and low concentration controls in ten analytical runs performed on different days. Analytical relative recovery was determined by duplicate analysis of high and low controls. Selectivity was determined by analysis of numerous alcohols, glycols and metabolites of ethylene glycol at a concentration of 1000 mg/l. A method correlation was carried out between the HPLC method and an "in house" gas chromatography assay using patient specimens.

2.7. Gas chromatography instrument and analytical conditions

Experimental, standard and control serum specimens (100 μl) were mixed with 100 μl of acetonitrile containing 1.0 mg/ml 1,3-propanediol as the internal standard. The analyses were performed on a Hewlett-Packard (Avondale, PA, USA) Model 5880 gas chromatograph outfitted with a 15 m×0.53 mm

I.D., 0.5 μm film thickness Supelco Nukol fused-silica capillary column (Bellefonte, PA, USA) and a flame ionization detector. The chromatographic conditions were as follows: injection port temperature 228°C, detector temperature 200°C, splitless injection, purge vent 2–4 ml/min and head pressure 550 kPa. Helium was the carrier gas with a flow-rate of 15 ml/min. The oven temperature was maintained at 110°C throughout the analysis. Hydrogen gas for the detector was maintained with a flow-rate of 20 ml/min. Retention times for ethylene glycol and internal standard, 1,3 propanediol, were 3.4 min and 7.1 min, respectively.

3. Results and discussion

The present study describes and validates an HPLC method for the measurement of ethylene glycol in plasma. This assay represents a modification of a previously reported HPLC method utilizing benzoyl chloride derivatization by means of the Schotten–Baumann reaction [8]. The assay is linear from 20–2000 mg/l (Fig. 1). The slope±standard error and intercept±standard error are 0.0028±0.0001 and −0.0022±0.0288, respectively. The correlation coefficient (r^2) is 0.997. The limits of detection (sensitivity) and quantitation are 10 mg/l and 20 mg/l, respectively (Fig. 1). Although the limit of detection for this assay is identical to that for the original HPLC method, this limit is achieved using only a 100-μl sample. This is five-fold less than the sample volume required for the original HPLC method (500 μl) [8]. The limit of quantitation for this optimized HPLC assay is five-fold better than that for the gas chromatography used in this study (100 mg/l). Optimized reaction and extraction conditions extend the linear range (20–2000 mg/l) relatively to that reported for the original method (25–400 mg/l) [8]. This extended linearity is of clinical importance as blood levels in excess of 500 mg/l represent one indication for hemodialysis [5]. This reduces the need to dilute patient specimens to achieve an analytical result within the dynamic range of the assay and thus avoids delayed reporting of results in patients with very high ethylene glycol levels.

Within- and between-run imprecision for the

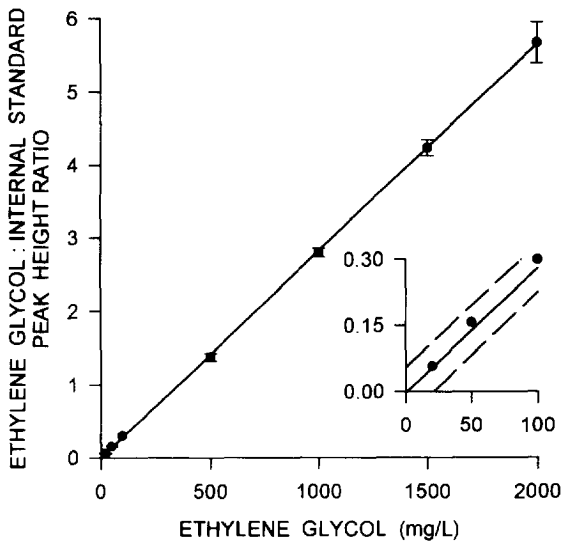

Fig. 1. Linearity of the calibration graph and limit of quantitation. The larger graph illustrates linearity of the calibration curve over a 20–2000 mg/l concentration range as defined by least squares fit regression. The slope, intercept and coefficient of determination (r^2) are listed in Section 3. Points and error bars represent the mean and standard deviation, respectively, for analysis of separate concentration standards (n=4 at each concentration). Where error bars are not evident, the standard deviation was less than the magnitude of the symbol. The solid line on the inset graph illustrates linearity at low concentrations. The dashed lines reflect the 95% confidence interval determined from the entire regression line. The limit of quantitation, 20 mg/l, corresponds to the lowest concentration for which the lower limit of the 95% confidence interval was greater or equal to a peak-height ratio of zero. Based on this definition, the limit of quantitation can be measured with 95% confidence. The limit of detection (sensitivity), 10 mg/l, was defined as a signal at least three times greater than background chromatographic noise.

modified method (≤9.5%, Table 1) was consistently better than provided by the initial HPLC assay (≤12%). Improved assay imprecision is due to use of a glycol internal standard (1,3-propanediol) rather than an alcohol internal standard (benzoyl alcohol). Improved extraction efficiency with solid-phase rather than liquid–liquid extraction also contributed to the lower assay imprecision. Solid-phase extraction has the added benefit of reduced sample preparation time. Not surprisingly, between-run imprecision was slightly greater than within-run imprecision.

Assay selectivity is based on analyte retention time and the retention time relative to that of the internal standard. Example chromatograms are

Table 1
Assay imprecision

Run	Control	Target (mg/l)	Mean (mg/l)	S.D. (mg/l)	C.V. (%)	Recovery (%)
Within	Low	20	20	1	5.0	100
	Medium	250	260	7	2.7	104
	High	1500	1470	24	1.6	98
Between	Low	20	21	2	9.5	105
	Medium	250	260	12	4.8	104
	High	1500	1500	36	2.4	100

Within- and between-run assay imprecision was based on repetitive analysis ($n=10$) of low, medium and high controls with the target concentrations designated above. The mean, standard deviation (S.D.), imprecision expressed as the coefficient of variation (C.V.) and the percent analytical recovery expressed as the mean concentration relative to the target concentration are shown.

shown (Fig. 2). Use of a 4 μm C_{18} column with end capping and a 50:50 acetonitrile–water mobile phase provided improved resolution and thus, greater specificity. The assay is free from interference by other common volatile glycols and alcohols (Table 2). Use of 1,3-propanediol as the internal standard is preferred to propylene glycol, as the latter is a constituent in numerous foods and drug preparations [9]. This has proven problematic when propylene glycol containing anticonvulsant drug preparations were used in the treatment of ethylene glycol poisoned patients. In particular, propylene glycol in patient specimens introduced error in the quantitation of ethylene glycol when the former was used as internal standard [9]. In our own laboratory, propylene glycol in patient specimens were misidentified as ethylene glycol when analyzed by an enzyme-based screening method. Confirmation analysis using HPLC correctly identified contaminant propylene.

This modified HPLC assay for ethylene glycol was developed for a laboratory without a gas chromatograph, generally accepted as the method of choice. Comparison of serum ethylene glycol levels for 17 patient specimens analyzed using this modified HPLC assay compared favorably with results obtained by gas chromatography when examined over a concentration range of 160–1510 mg/l. The correla-

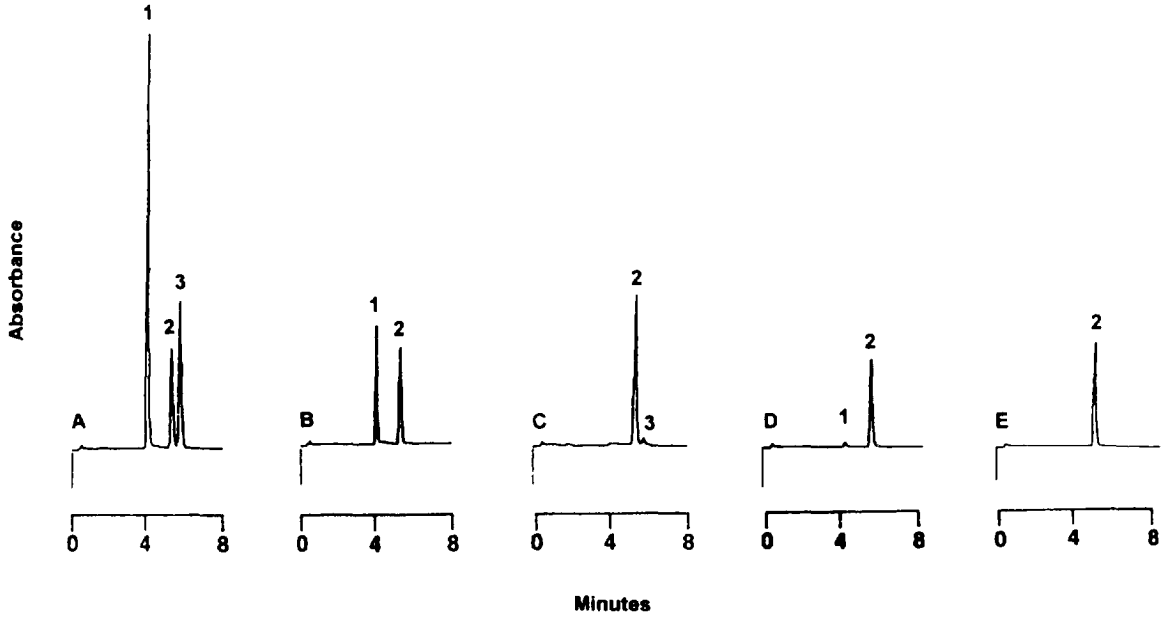

Fig. 2. Representative HPLC chromatogram. (A) Representative HPLC chromatogram from the analysis of a mixed standard prepared in bovine serum and containing 1500 mg/l ethylene glycol (1), internal standard (2) and 1000 mg/l propylene glycol (3). (B) Chromatogram from the analysis of a patient sample positive for ethylene glycol (1) at 440 mg/l and with added internal standard (2). (C) Chromatogram from the analysis of a patient specimen with added internal standard (2) and positive for propylene glycol (3) at 40 mg/l. (D) Chromatogram from the analysis of a patient sample positive for ethylene glycol (1) at 20 mg/l and with added internal standard (2). (E) Chromatogram from the analysis of a patient sample negative for ethylene glycol and with added internal standard (2).

Table 2
Assay selectivity

Analyte	Retention time (min)	Relative retention time[a]	k'
Acetone	n.d.		
1,3-Butanediol	8.3	1.4	15.6
2,3-Butanediol	8.4	1.4	15.9
1-Butanol	n.d.	0.2	
2-Butene-1,4-diol	8.3	1.4	15.7
Ethanol	n.d.		
Ethylene glycol	4.3	0.7	7.7
Glucose	n.d.		
Glycerol	15.3	2.6	29.8
Glycolic acid	n.d.		
Glyoxylic acid	n.d.		
Methanol	n.d.		
3-Methyl-1-butanol	n.d.		
Oxalic acid	n.d.		
2,4-Pentanediol	11.1	1.9	21.4
2-Propanol	n.d.		
1,2-Propanediol	6.2	1.0	11.5
1,3-Propanediol	5.7	1.0	10.5

[a] Analyte retention time expressed in minutes, analyte retention time relative to the retention time of the internal standard (1,3-propanediol), and analyte retention expressed as the capacity factor (k') equal to the retention time of the retained analyte minus the time for elution of unretained solute divided by the time for elution of unretained solute. Several analytes were not detected (n.d.).

tion coefficient (r^2) for least-squares fit linear regression analysis is 0.998. The slope±standard error and intercept±standard error are 0.979±0.022 and 9.78±0.78, respectively. Positive bias for the optimized HPLC assay relative to the gas chromatography method is due, in part, to the five-fold higher limit of quantitation for the latter assay relative to the former. Despite generally good correlation with gas chromatography, this HPLC assay has two drawbacks. First, HPLC requires the use of relatively expensive glass distilled organic solvents. Second, preparation of patient specimens for analysis by HPLC requires more time than preparation of the same specimens for gas chromatography. For example, preparation of a single calibrator, two controls, a blank and a single patient specimen for HPLC requires approximately 40 min. By contrast, preparation of the same specimens for gas chromatography requires only 7 min. The time for chromatographic analysis by both methods is the same, 8 min per specimen. Because HPLC requires more time, we routinely screen patient specimens for ethylene glycol using a glycerol dehydrogenase-based method developed for the DuPont aca [2]. The HPLC assay is used as a confirmatory method and to monitor clearance of ethylene glycol during ethanol administration and/or hemodialysis. When not in use, the HPLC system is maintained in the mobile phase, 50:50 acetonitrile–water. As a result, there is no (or very little) time needed to equilibrate the column either before or after ethylene glycol analysis. Finally, the HPLC assay does not suffer from the type of carry over commonly encountered in many gas chromatographic methods for ethylene glycol.

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