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Gas chromatographic–mass spectrometric identification and quantitation of ethylene glycol in serum after derivatization with perfluorooctanoyl chloride: a novel derivative

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Abstract

Ethylene glycol poisoning is a common clinical problem and identification as well as quantitation of ethylene glycol in serum is important for medical and legal purposes. Most investigators described determination of ethylene glycol by gas chromatography without derivatization or derivatives forming a molecular ion <200. We describe a novel derivatization technique of ethylene glycol using perfluorooctanoyl chloride, after extraction from serum using acetone. This derivative has a molecular mass of 854 and produces a base peak at m/z 441 and other diagnostic strong peaks for unambiguous identification. Moreover, this derivative is less volatile and is free from interferences from endogenous serum components. Quantitation can be achieved by using 1,4-butanediol as an internal standard. The assay showed within-run and between-run precision of 7.2% and 8.0%, respectively, and linearity over the serum ethylene glycol concentration range 70–2240 μg/ml with a detection limit of 5 μg/ml.

1. Introduction

Human intoxication with ethylene glycol is a common problem and occurs in both clinical and forensic toxicology due to wide availability of ethylene glycol as an antifreeze and windshield washer formula. As with methanol and isopropanol, ethylene glycol is commonly ingested by misguided or debilitated alcoholics. Severe intoxication from ethylene glycol can cause death and determination of ethylene glycol in blood is routinely performed in most clinical and forensic laboratories.

There are several methods for determination of ethylene glycol in serum or blood including gas chromatography [1–6], gas chromatography–mass spectrometry [7] and enzymatic assay [8,9]. Most gas chromatographic techniques involve direct determination of underivatized ethylene glycol and suffer from problems associated with tailing of the peaks, sensitivity and ghosting [10]. Peterson and Rodgerson [11] described a method for gas chromatographic assay of ethylene glycol after converting it to the corresponding dibenzoate ester, but the technique is tedious and produced low sensitivity [12]. Another fluorometric measurement of ethylene glycol where ethylene glycol was dehydrogenated by sodium

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periodate to form two molecules of formaldehyde, which was then combined with ammonium ion and acetylacetone to form fluorescent acetyllutidine is also technically difficult [13]. Moreover, in the gas chromatographic technique, ethylene glycol is identified based on retention time only and interferences from other glycols are possible. Jones et al. [14] recently reported a case where 2,3-butanediol was mistakenly identified as ethylene glycol in a routine toxicological analysis using a gas chromatographic technique. This can be specially problematic in a forensic laboratory where the results may involve serious legal issues. Needham et al. [15] reported derivatization of ethylene glycol with bromophenyl boronic acid, Porter and Auansakul [16] with phenyl boronic acid, Robinson and Reive [12] with *n*-butyl boronic acid and Maurer and Kessler [7] with pivalic acid. Poole [17] reported that derivatization with substituted boronic acid which forms cyclic ester with 1,2-diols, can not be used for diethylene glycol, e.g. 1,4-butanediol, due to the greater distance between the two hydroxyl groups. Therefore, pivalic acid derivatives can be used for any glycols. However, the mass spectra of the pivalate derivative of ethylene glycol did not produce a molecular-ion peak and the strongest peaks were observed only at *m/z* 57 and *m/z* 85 [14]. The phenylboronate derivative of ethylene glycol produced strong peaks at *m/z* 148, 118 and 91. A new derivatization technique of ethylene glycol which could lead to less volatile derivatives and produce strong peaks at a much higher mass range could be useful for clinical and especially for forensic laboratories for unambiguous confirmation of ethylene glycol in human serum. In the present paper we report our protocol for identification and quantitation of ethylene glycol as its perfluorooctanoyl derivative which produced a base peak at *m/z* 441 in the mass spectrum.

2. Experimental

Ethylene glycol, 2,3-butanediol and 1,4-butanediol were purchased from Sigma (St. Louis, MO, USA). The derivatizing reagent perfluorooctanoyl chloride was obtained from PCR Chemicals (Gainsville, FL, USA). The derivatization reactions were carried out in disposable 5-ml screw-capped conical test tubes. 1,4-Butanediol was used as an internal standard. The internal standard was dissolved in acetone to produce a final concentration of 500 μg/ml.

For extraction, 50 μl of serum or plasma was transferred to a small plastic 1.5-ml microcentrifuge tube and 50 μl of acetone containing the internal standard was added (500 μg/ml). The sample was vortex-mixed and allowed to stand for 2–3 min. Then the sample was centrifuged at a very high speed (16 000 *g*, Eppendorf Centrifuge, Model 5415 C) for 4 min and the clear supernatant was transferred to a disposable 5-ml screw-capped conical test tube. The solvent was evaporated under air at room temperature and then 50 μl of derivatizing reagent was added. The reaction mixture was incubated at 60°C for 20 min in a temperature controlled heating block. After incubation, the excess derivatizing reagent was evaporated under air and the dry residue was reconstituted with 1 ml of ethyl acetate. If necessary, the sample was centrifuged at 1500 *g* for 5 min and 1–2 μl of the clear supernatant was injected onto the gas chromatography–mass spectrometer system.

The gas chromatographic–mass spectrometric (GC–MS) analysis was carried out using a Model 5890 gas chromatograph coupled with a 5970 Series mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). The capillary column used was an Ultra-2, 25 m × 0.20 mm I.D. (Hewlett-Packard). The column was coated with phenyl methylsilicone (film thickness 0.33 μm). The column head pressure of helium gas was 69 kPa with a linear velocity of 31.5 cm/s. A manual injection technique was employed with an injector temperature of 200°C. The initial oven temperature of the gas chromatograph was 80°C. After maintaining that temperature for 2 min, the temperature was increased at a rate of 4°C/min to reach an oven temperature of 130°C. Then the oven temperature was increased at a rate of 15°C/min to reach a final oven temperature of 270°C. The mass spectrometer was scanned from *m/z* 40 to *m/z* 500 and the solvent

delay was 10 min. The identification of ethylene glycol was performed by matching the reference mass spectrum with the mass spectrum of ethylene glycol observed in patient serum after extraction and derivatization. Chromatography was not performed in the selected-ion mode because the concentrations of ethylene glycol usually encountered in patient sera are sufficient to produce good quality mass spectra in the scan mode. Quantitation was done by comparing the peak area of ethylene glycol with the peak area of internal standard. The peak areas were measured in the total-ion chromatogram. We constructed a calibration table and considered the peak as the ethylene glycol peak if the retention time was within ± 0.15 min of the expected retention time of ethylene glycol. The same criteria were also applied for the internal standard peak.

The fast-atom bombardment mass spectrometric study was performed using a Model VG 70-SEQ mass spectrometer manufactured by Varian Instruments. The matrix used for this study was 3-nitrobenzoyl alcohol. The samples were bombarded with xenon atom beams with 8 keV energy.

The concentration of ethylene glycol in serum was also measured by an enzymatic assay using glycerol dehydrogenase from *Enterobacter aerogenes* which has a high specificity for ethylene glycol. The assay was performed on a Monarch 2000 centrifugal analyzer (Instrumentation Laboratory, Lexington, MA, USA) as described previously [18].

3. Results and discussion

3.1. Choice of internal standard

We chose 1,4-butanediol as an internal standard instead of the more commonly used 2,3-butanediol because of the recent report of Jones et al. [14] where 2,3-butanediol was mistakenly identified as ethylene glycol by gas chromatography. Moreover, the technique used in the present study can also derivatize 1,4-butanediol while the conventional technique of forming cyclic esters does not work with 1,4-diols. We observed very good separation between derivatized ethylene glycol (retention time 13.2 min), 2,3-butanediol (retention time 13.8 min) and the internal standard, which had the longest retention time of 17.3 min.

3.2. Precision and recovery

The within-run and between-run precision of the assay were determined by using a serum standard containing 560 μg/ml of ethylene glycol. The coefficient of variation (C.V.) of the within-run precision was 7.2% ($X = 563.0$, S.D. = 41.0, $n = 10$) while the between-run precision was 8.0% ($X = 536.0$, S.D. = 43.1 $n = 10$).

Acetone extraction of serum was performed according to Maurer and Kessler [7], except that we used 50 μl of serum and 50 μl of acetone and the supernatant was evaporated at room temperature instead of at 70°C. Attempts to evaporate the supernatant at 70°C lead to a lower recovery of ethylene glycol (46–65%) and therefore room temperature was used for evaporation of the solvent which requires approximately 15–20 min. Serum standards containing 0, 70, 140, 280, 560, 1120 and 2240 μg/ml of ethylene glycol were used for the recovery study. After extraction and evaporation of the solvent, ethylene glycol was derivatized along with the internal standard and the peak areas were compared with a similar standard of ethylene glycol prepared in acetone without extraction. A 74–81% recovery was found for ethylene glycol.

In another set of experiments, the serum standards of ethylene glycol were extracted with acetone (no internal standard) and after evaporation of solvent, the dry residues were reconstituted with serum containing no ethylene glycol and assayed by the enzymatic assay using a Monarch centrifugal analyzer. Recoveries of 72–86% for the serum standards were found (Table 1).

3.3. Linearity and detection limit

The assay was linear for the serum ethylene glycol concentration range 70–2240 μg/ml.

Table 1
Recovery of ethylene glycol from serum standard after extraction using the enzymatic assay

Standard	Ethylene glycol concentration (μg/ml)		
	Target	Observed	Recovery (%)
Standard 1	70	65	86.6
Standard 2	140	100	71.4
Standard 3	280	211	75.4
Standard 4	560	466	83.2
Standard 5	1120	922	82.3
Standard 6	2240	1940	85.7

Linear regression analysis using the x-axis as the target concentration and the y-axis as the observed concentration by the GC–MS assay, produced the following regression equation: $y = 1.11x - 24.93$. The correlation coefficient was 0.98. The mass spectrometer was calibrated every day and the solution of internal standard in acetone was renewed every week. The range of our assay is wider than that of the automated enzymatic assay for ethylene glycol which showed linearity over the ethylene glycol concentration range 100–1120 μg/ml. The detection limit of the assay was 11.0 μg/ml of ethylene glycol in serum, which is substantially lower than the detection limit of 30 μg/ml obtained with the enzymatic assay. The detection limit can be lowered to 5.0 μg/ml if the dry residue after derivatization of ethylene glycol was reconstituted with 100 μl of ethyl acetate instead of 1 ml of ethyl acetate. Our GC–MS assay showed no carry over: when serum negative for ethylene glycol was assayed just after assaying a serum standard containing 2240 μg/ml of ethylene glycol, no ethylene glycol peak was found in the blank serum and only the internal standard peak was present. When ethylene glycol in serum is analyzed by direct injection, the peak may be sharp without tailing, but may show a "memory effect", i.e. injection of water just after a serum specimen that contained ethylene glycol resulted in the elution of a substantial ethylene glycol peak [16].

3.4. Stability of derivative

Derivatized ethylene glycol is susceptible to hydrolysis. We observed approximately 10–13% loss in 4 h when the derivative was stored at room temperature. The rate of hydrolysis can be significantly reduced if the derivative was stored at 4°C. We observed only 20% loss in 72 h.

3.5. Analysis of patient sera

Comparable results for the ethylene glycol concentrations in serum of seven patients were obtained with the enzymatic assay and the new GC–MS assay presented here (Table 2). The linear regression analysis using the concentration observed by the enzymatic assay as the x-axis and the concentration observed by the GC–MS assay as the y-axis produced the following regression equation: $y = 0.946x + 23.37$. The correlation coefficient was 0.91. The enzymatic assay on the Monarch 2000 centrifugal analyzer for ethylene glycol is routinely used in our clinical toxicology laboratory for patient monitoring. The performance of this assay is already well established and we used it as a reference method. Moreover, different serum standards containing ethylene glycol gave comparable results by both the GC–MS assay and the enzymatic assay. The serum blank or zero standard did not produce any ethylene glycol peak. Moreover, other serum specimens containing no ethylene glycol did not produce any peak for ethylene glycol in our GC–MS assay, indicating that endogenous

Table 2
Comparison of ethylene glycol concentrations in patient sera by GC–MS and the enzymatic assay

	Ethylene glycol concentration (μg/ml)	
	Enzymatic assay	GC–MS assay
Patient 1	405	417
Patient 2	219	246
Patient 3	326	307
Patient 4	240	303
Patient 5	237	223
Patient 6	213	196

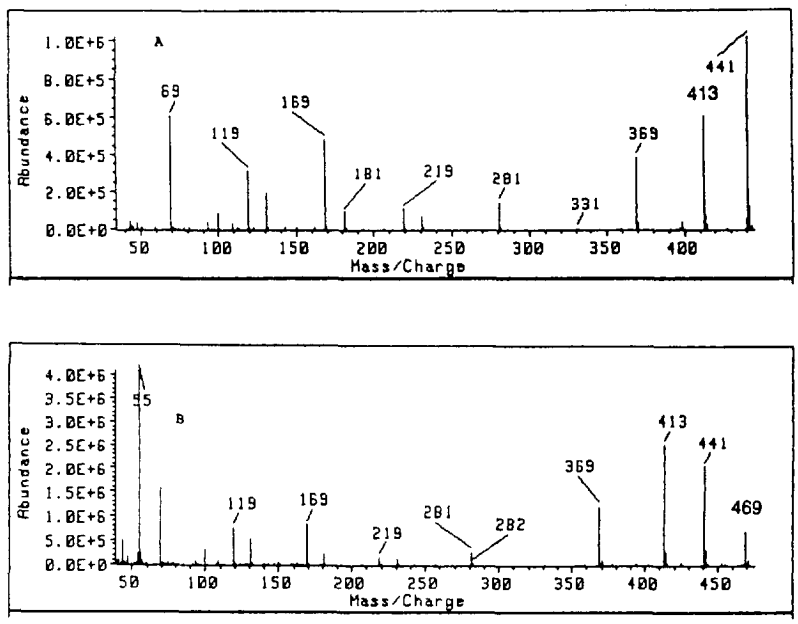

Fig. 1. Electron-impact mass spectral fragmentation pattern of perfluorooctanoyl derivatives of (A) ethylene glycol, (B) 2,3-butanediol.

serum components do not interfere with our assay.

3.6. Mass spectral characteristics

The major advantage of our GC–MS assay is the mass spectral characteristics of ethylene glycol which lead to unambiguous confirmation of ethylene glycol in human serum. The base peak of the perfluorooctanoyl derivative of ethylene glycol was observed at m/z 441 (Fig. 1). Two other strong peaks were observed at m/z 413 (relative abundance 54%) and m/z 369 (relative abundance 33.6%). Since we observed no peaks in the mass range 500–800, the scanning range of the mass spectrometer was set at m/z 40–500. The perfluorooctanoyl derivative of ethylene glycol extracted from patient sera produced mass spectral characteristics as expected. The proposed mass spectral fragmentation pattern of this new derivative of ethylene glycol is given in Fig. 2.

The mass spectral characteristics of the perflurooctanoyl derivative of 2,3-butanediol is different from that of the derivatized ethylene glycol. In this case the base peak was observed at m/z 55. Another diagnostic peak was observed at m/z 469 (relative abundance 17%) as expected due to the addition of two methylene groups in the molecule (Fig. 1). Moreover, the perfluorooctanoyl derivative of 2,3-butanediol had a longer retention time than ethylene glycol

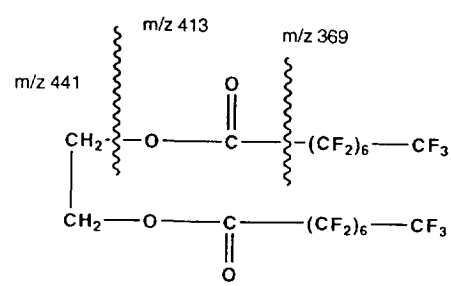

Fig 2. Proposed fragmentation pattern of the perfluorooctanoyl derivative of ethylene glycol.

but a shorter retention time than the internal standard. Therefore, our assay can distinguish ethylene glycol from 2,3-butanediol.

The expected molecular mass of the perfluorooctanoyl derivative of ethylene glycol is 854, if both hydroxyl groups are derivatized. The mass spectrometer used in the GC–MS assay can not scan beyond m/z 800 and therefore we were unable to observe any molecular ion. In order to ensure that under the conditions used both hydroxyl groups are completely derivatized, we studied the molecular structure of our derivative by fast-atom bombardment mass spectrometry (FAB). In this mode we observed a weak molecular ion at m/z 854 and a slightly stronger M − 1 ion at m/z 853. Again the base peak was observed at m/z 441. Other characteristics peaks at m/z 413 and 369 were also observed. When the perfluorooctanoyl derivative of the internal standard was studied by FAB-MS, the M − 1 peak was shifted to m/z 881 due to the addition of two methylene groups as expected. We also observed a weak molecular ion peak at m/z 882. The base peak was also shifted to m/z 469 as expected (Fig. 3). We therefore conclude that our technique produced the desired derivatives of ethylene glycol and the internal standard, where both functional hydroxyl groups are derivatized.

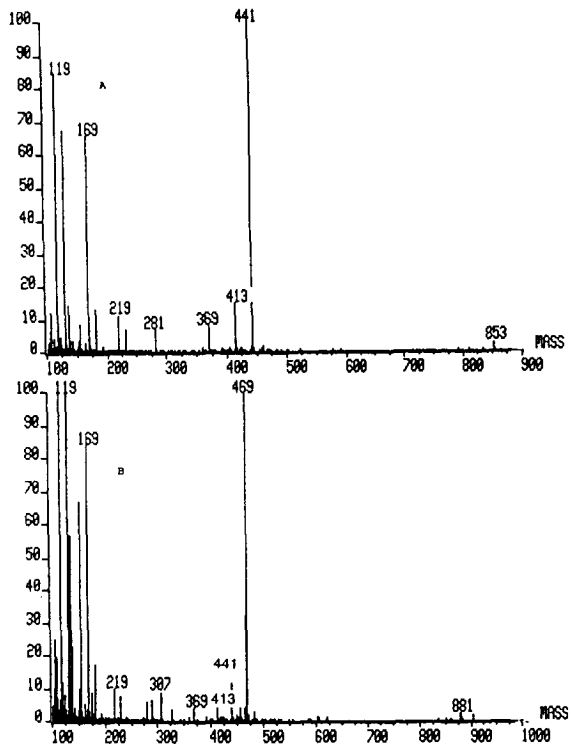

Fig 3. Fast-atom bombardment mass spectral fragmentation pattern of perfluorooctanoyl derivatives of (A) ethylene glycol, (B) internal standard.

3.7. Application of the assay

Enzymatic assays for ethylene glycol are widely used in clinical laboratories due to simplicity of operation and speed. However, enzyme-based assays lack absolute specificity especially when faced with legal challenges. There are other reports on the determination of ethylene glycol in serum or plasma using gas chromatography [19], but molecular identification again is not possible. Other glycols, for example 2,3-butanediol can be mistakenly identified as ethylene glycol by gas chromatography. Maurer and Kessler [7] reported a GC–MS identification and quantitation of ethylene glycol after derivatization with pivalic acid anhydride, but their derivative for ethylene glycol produced a base peak at m/z 57, another strong peak at m/z 85 and a weak peak at m/z 143. The derivatization technique used in the present assay produced diagnostic peaks in the much higher mass range, with a base peak at m/z 441 and other strong peaks at m/z 369 and 413, which are certainly useful in the unambiguous confirmation of ethylene glycol in human serum. Moreover, the mass spectrum of derivatized ethylene glycol is distinctly different from that of other glycols. The retention times of derivatized ethylene glycol and the internal standard were 13.1 min and 17.0 min under the gas chromatographic conditions employed (Fig. 4). Our less volatile derivatives did not show any interferences from other alcohols like methanol, ethanol, isopropanol or other endogenous volatile components in serum.

Our assay would be useful in forensic toxicology laboratories where the results have serious legal implication. For legal drug testing the GC–MS standard is considered as gold standard. Our

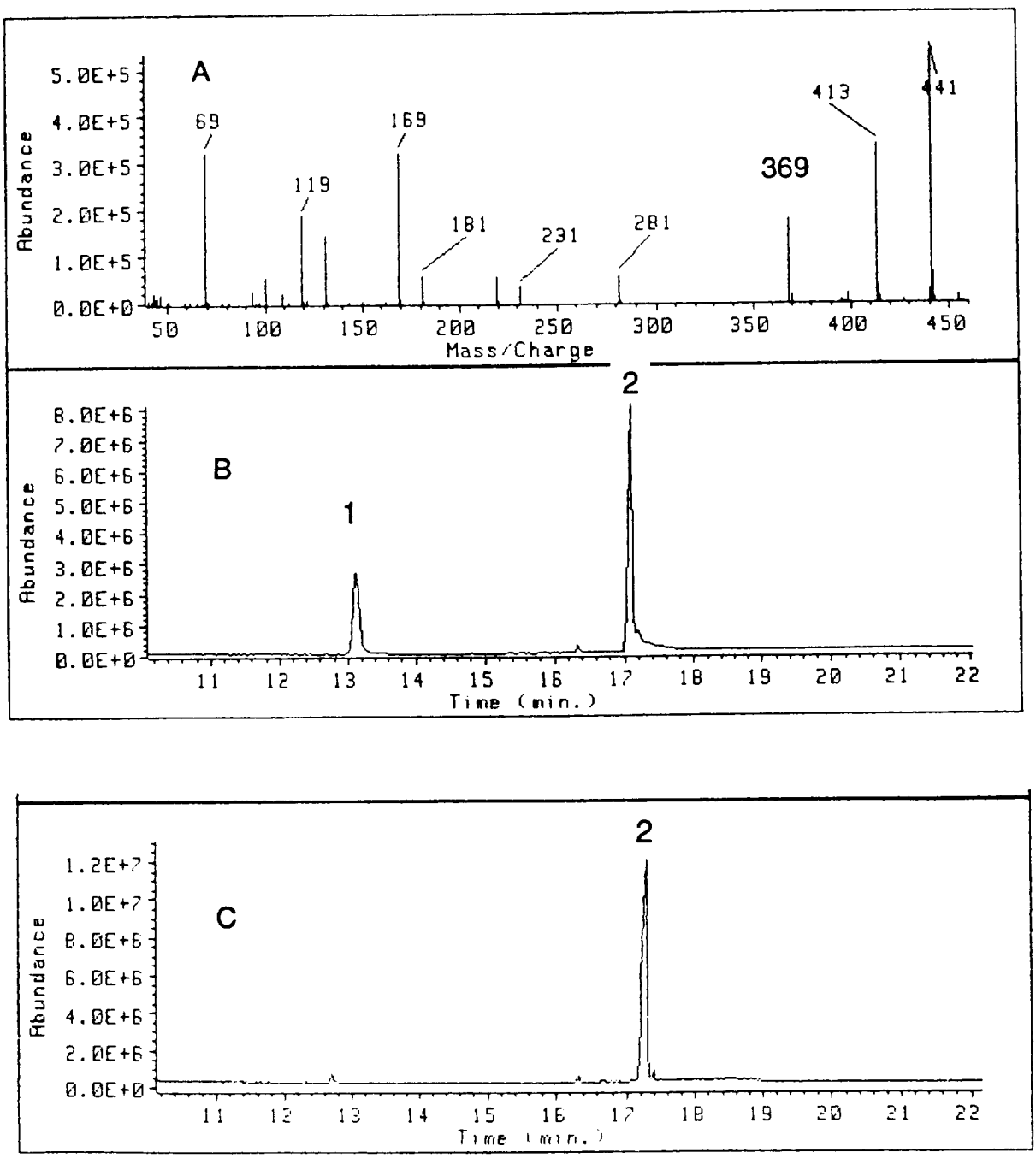

Fig. 4. (A) Mass spectrum of peak 1 indicating the presence of ethylene glycol in patient serum. (B) Total-ion chromatogram of a patient serum containing 213 μg/ml of ethylene glycol. Peak number 1 represents ethylene glycol while peak number 2 is the internal standard. (C) Total-ion chromatogram of a patient serum containing no ethylene glycol. Peak number 2 represents the internal standard.

GC–MS technique for confirmation of ethylene glycol could be useful for establishing the cause of death by ethylene glycol overdose. Our assay may also be useful in clinical toxicology laboratories where a higher specificity of the assay is desired for confirmation of ethylene glycol, especially when confusion arises with respect to the presence of other glycols by a routine GC assay without any derivatization or when carry-over problems occur from a previous run containing a high concentration of ethylene glycol in the serum.

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