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Short communication

Fast determination of ethylene glycol, 1,2-propylene glycol and glycolic acid in blood serum and urine for emergency and clinical toxicology by GC-FID

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ABSTRACT

A simple, cost effective, and fast gas chromatography method with flame ionization detection (GC-FID) for simultaneous measurement of ethylene glycol, 1,2-propylene glycol and glycolic acid was developed and validated for clinical toxicology purposes. This new method employs a relatively less used class of derivatization agents – alkyl chloroformates, allowing the efficient and rapid derivatization of carboxylic acids within seconds while glycols are simultaneously derivatized by phenylboronic acid. The entire sample preparation procedure is completed within 10 min. To avoid possible interference from naturally occurring endogenous acids and quantitation errors 3-(4-chlorophenyl) propionic acid was chosen as an internal standard. The significant parameters of the derivatization have been found using chemometric procedures and these parameters were optimized using the face-centered central composite design. The calibration dependence of the method was proved to be quadratic in the range of 50–5000 mg mL^{-1}, with adequate accuracy (92.4–108.7%) and precision (9.4%). The method was successfully applied to quantify the selected compounds in serum of patients from emergency units.

1. Introduction

Ethylene glycol (EG) poisoning is a relatively frequent medical emergency, may result in significant morbidity and mortality if untreated [1,2], and represents challenges both for clinicians and toxicological laboratories. Ethylene glycol, an odorless dihydric alcohol, is a sweet-tasting ingredient in antifreeze solutions, brake fluids and other easily reachable household preparations. EG poisonings result from either unintentional, or suicidal ingestions, although intentional criminal EG poisoning have been reported [3]. 1,2-Propylene glycol (PG) is according to FDA 'generally recognized as safe' and is a common component of many pharmaceuticals and food additives, but fatal overdosing have been also reported [4]. There have been 160 requests from emergency units for determination of ethylene glycol for differential diagnostic procedure in our laboratory in 2013, from which 33 cases were positive.

EG is metabolized to glycol aldehyde with rapid subsequent conversion to glycolic acid (GA). Thus, measurement of serum ethylene glycol, and ideally GA, its major toxic metabolite in serum, is definitive. GA is on the other hand slowly oxidized to glyoxylic acid, which is normally converted to glycine by alanine-glyoxylate aminotransferase (AGT), but in EG poisoning conditions AGT becomes saturated and results in increased oxalate formation [5]. Calcium oxalate crystals may be deposited in renal tubules causing acute renal failure and may also contribute to other organ toxicity. GA is the principle toxic metabolite in serum and its concentrations correlate with severity of poisoning unlike that for EG serum concentrations [6]. Metabolic acidosis develops shortly after EG ingestion and is a vital sign of EG intoxication along with high anion gap and osmolal gap [7,8]. These indicators are unfortunately highly non-specific and could be attributed to other conditions, for instance diabetic ketoacidosis [9] or multiple organ failure [10]. EG poisoning treatment is performed by either antidote theraphy, or hemodialysis, or combination of them. The used antidotes are ethanol and fomepizole, but the second one is not commonly available in the Czech Republic. The antidote treatment of EG and PG is recommended at serum concentration starting at 200 mg L^{-1} and 1000 mg L^{-1}, respectively [11,12]. The detailed overview and discussion about thresholds for toxicity and antidote theraphy can be found in a review of Porter [2].The simultaneous measurement of toxic GA along with parent EG is clearly desirable for both enhanced clinical service and laboratory correct diagnosis. Unfortunately, there is a lack of appropriate

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methods for simultaneous determination of both analytes. The already published GC methods for simultaneous determination involve derivatizating step with different kinds of silylating agents because of their capability to react with all active hydrogens present in the molecule of analyte, i.e. hydroxy and carboxylic –OH group, thus the derivatization proceeds in one step. GC-FID methods have been employed for this analytical challenge firstly [13]. Nowadays, the most reliable simultaneous determination of EG and GA is based on GC–MS procedures, mostly employing silanization agents for derivatization [14,15]. Alternative liquid chromatography or electrophoretic methods have been conducted, but mostly for metabolites only [16,17].

The goal of this study was to develop fast, reliable and simple method that is not based on silanization for simultaneous determination of EG, PG and GA in blood serum or urine by GC-FID. The use of GC-FID instrument is advantageous over GC–MS (although our GC-FID method could be easily transferred to GC–MS method if desired) because the GC-FID instrumentation is widespread due its low cost, simple maintenance and the possibility of the use of hydrogen instead of helium as a carrier gas. Furthermore, GC-FID instrument could be dedicated to this method solely and operated by well-trained technical staff without supervision on 24 h daily service.

In our method we have adopted previously published method developed in our department during boronate ester era in late 80 s [18]. This method is based on reaction of glycols with phenylboronic acid. However, this procedure is not suitable for determination of glycolic acid since phenylboronic acid readily reacts with some diols only. For this reason, we have integrated another parallel derivatization step employing relatively unusual derivatization agent – alkylchloroformates, which have been applied for multiple analytes and matrices [19–23]. The common drawbacks of aforementioned silanization procedures are necessity of water-free reaction medium, sample heating and finally the high cost of the silanization agents. On the contrary, alkylchloroformates react readily in aqueous medium in seconds in high yields. They react with moieties with active hydrogen, e. g. carboxylic acids, phenols, and amines yielding the corresponding derivatives (carboxylic acid esters, carbonic acid diesters and carbamides). The scheme of derivatization reaction of GA with isobutyl chloroformate is shown in Fig. 1.

Additionally, there has been a discussion about the cost and volumes of silylation agents routinely used in toxicological laboratories for determination of glycols, which has led us to find an economical alternative [15,24]. Concerning our laboratory, a request for a suspected glycol intoxication confirmation is a daily routine. Price of volumes of derivatization reagents used in our proposed method, i.e. phenylboronic acid and isobutyl chloroformate, are several fold cheaper compared to the price of BSTFA amounts used in published papers [2,14,15,24]. Furthermore, the complete sample preparation time is shortened to nearly 10minutes and there is no need for any sample heating or microwave irradiation. The quantitative serum results are available within 30 min, which correspond with the usual emergency medicine turnaround time. However, the phenylboronic acid is not suitable for derivatization of diethylene, triethylene and tetraethylene glycol, but intoxications with these compounds are rather rare in Europe. Nevertheless, the measurement of metabolites of these rare compounds could be implemented to overcome this limitation i.e. diglycolic acid and 2-hydroxyethoxyacetic acid [25].

The optimal conditions of derivatization method have been found using chemometric approach (fractional factorial design and central composite design), replacing the common one-factor-at-a-time (OFAT) procedures because they are not time effective and do not take into the account possible interactions among the individual parameters.

2. Material and methods

2.1. Chemicals and material

Ethylene glycol (p.a.), ethanol (p.a.) and acetic acid (p.a.) were purchased from Penta (Czech Republic). Glycolic acid (99%), methanol (Chromasolv), phenylboronic acid (97%) and di-sodium hydrogen phosphate dihydrate (p.a.) were purchased from Fluka (Germany). 1,2-propylene glycol (99.5%), 1,3-propylene glycol (98%), 3-(4-chlorophenyl) propionic acid (97%), pyridine (99.8%), isobutanol (99%), methyl chloroformate (99%), ethyl chloroformate (97%), isobutyl chloroformate (98%), sodium acetate (99%), boric acid (p.a.), potassium chloride (p.a.) and sodium carbonate (p.a) were purchased from Sigma-Aldrich (Germany). Hexane (Li Chrosolv) was purchased from Merck (Germany).

2.2. Preparation of serum and urine samples, calibrators

Blank human serum for method validation was purchased from ACQ Science GmbH (Germany). Blank urine samples were provided by three healthy male and three healthy female volunteers from our department and stored at $+4\ ^{\circ}C$ until use.

The seven concentration levels of the calibrators in blank human serum/urine were prepared to yield the following final serum concentrations for EG, PG and GA: CA1: 25 mg L^{-1}, CA2: 50 mg L^{-1}, CA3: 100 mg L^{-1}, CA4: 250 mg L^{-1}, CA5: 1000 mg L^{-1}, CA6: 2000 mg L^{-1}, CA7: 5000 mg L^{-1}.

Human whole blood or urine samples were delivered from various hospitals with request for glycols quantification, mainly for emergency purposes. Whole blood was centrifuged at 10,000 rpm for 30 s to obtain serum sample for analysis.

2.3. Sample pretreatment

In 1.5 mL Eppendorf tube, 100 μL of serum or urine was mixed with 100 μL of borate buffer of pH 9.0 (0.1 mol L^{-1}). Then 30 μL of internal standards (IS1: 1,3-propylene glycol, IS2: (3-(4-chlorophenyl) propionic acid); both 0.01 mg L^{-1} in isobutanol), 50 μL of isobutanol and 10 μL of pyridine were added and the mixture was shaken for 10 s. Then 100 μL of phenylboronic acid solution (0.003 mg L^{-1}, in isobutanol) was added and put into ultrasonic bath for 1 min. Finally, 50 μL of isobutyl chloroformate was added and the mixture was carefully mixed and sonicated for 5 min. Then the sample was centrifuged for 30 s (10,000 rpm) and 100 μL of upper isobutanol layer was transferred into 400 μL glass insert placed in 1.5 mL glass vial, and the vial was gas-tight crimped.

2.4. Instruments and apparatus

All analyses were performed on Shimadzu GC-2010 Plus with AOC-20i autosampler (Kyoto, Japan) and operated by a computer running GC solution version 2.41.00 SU1 (Kyoto, Japan). The

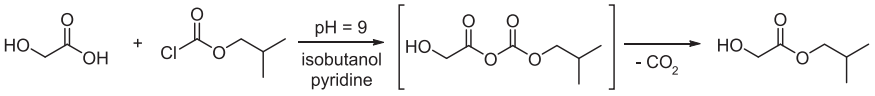

Fig. 1. Scheme of derivatization reaction of glycolic acid with isobutyl chloroformate.

chromatograph was equipped with Rtx-5 fused silica column (30 m × 0.25 mm ID, 0.50 μm film thickness, Restek, USA) coated with 5% diphenyl dimethyl polysiloxane. Hydrogen (purity 99.9992%) was employed as carrier gas. The oven temperature was held at 70 °C for 0 min following injection and then raised to 300 °C at 20 °C min^{-1}, total run time was 13 min. Instrument parameters were as follows: 250 °C inlet temperature, 310 °C detector temperature, column flow 2.82 mL min^{-1}. All injections were done in the split mode (1:50) and the volume of injected sample was 1 μL.

Micro-shaker type 326 m was from Premed (Warsaw, Poland) and ultrasonic bath type Transsonic T 310 was from Elma (Germany).

2.5. Statistical software

The construction and analyses of the experimental design and the response surfaces were carried out using the Minitab 16 statistical package (Minitab Inc., USA). Calculations were performed with Microsoft Excel 2007 (Microsoft, WA, USA). Chromatogram was constructed in Origin 9.1 (OriginLab Corporation, MA, USA).

2.6. Statistical evaluation of derivatization protocol

The parameters of optimization protocol for developed method (pH value, volumes of isobutanol, pyridine, isobutyl chloroformate and phenylboronic acid, concentration of phenylboronic acid and sonication times) have been selected on the basis of the literature and the own experience of authors [26,27] (Table 1).

The maximum response, defined as the absolute peak area of the selected analytes (EG, PG and GA), has been sought.

A constrain of the maximal applicable volume of reaction mixture is set to 650 μL, which condition is sufficiently fulfilled by Eppendorf vial of 1500 μL volume.

To screen the chosen parameters for isobutyl chloroformate derivatization protocol, the fractional factorial design has been used [28]. Table 1 summarized the proposed levels of these parameters: the low, high and the central ones. The Minitab 16 software proposed 33 experimental runs, combining the selected parameters. The results have been evaluated using the ANOVA, determining the main effects and interactions at a significant level of 95% [29,30]. The significant derivatization parameters for GA have been identified as pH, sonication time 1 min, volume of phenylboronic acid, volume of isobutanol, volume of isobutyl chloroformates, and concentration of phenylboronic acid. The significant parameters and some of their interactions are shown in Supplementary data (see Fig. S1.).

The only significant derivatization parameter for EG and PG has been identified as concentration of phenylboronic acid.

Table 1
Fractional factorial design parameters for optimization.

	Parameter	Level		
		Low	Central	High
A	pH	4	6.5	9
B	Volume of isobutanol (μL)	50	125	200
C	Volume of pyridine (μL)	10	40	70
D	Volume of isobutyl chloroformate (μL)	10	30	50
E	Sonication time (1) (min)	1	2	3
F	Volume of phenylboronic acid (μL)	20	60	100
G	Concentration of phenylboronic acid (mg mL^{-1})	0.1	1.55	3
H	Sonication time (2) (min)	1	3	5

To find the optimal values of the operational parameters (Table 1), the face-centered central composite design has been employed on the same data set as for the screening [31,32] are shown in Supplementary data (see Table S1).

3. Results

3.1. Method optimization

The alkyl chloroformates react with carboxylic acids in presence of corresponding alcohol and pyridine, yielding appropriate carboxylic acid esters [33]. The methyl-, ethyl- and isobutyl chloroformates were tested as agents for derivatization of glycolic acid. Methyl and ethyl esters of GA were extracted after derivatization with hexane, but polar hydroxyl group in GA molecule (see Fig. 1) probably caused insufficient extraction. Contrary to methanol and ethanol, isobutanol is not miscible with water and thus it can be used as extraction solvent. Additionally, it exhibits better partition coefficient (isobutanol/water) of glycolic acid isobutyl esters. Consequently, isobutanol was used in reaction mixture as precipitation and corresponding alcohol reaction agent, solvent for all analytes and internal standards.

Based on the statistical procedure applied (see text above) the optimal conditions were found as: pH 9, volume of isobutanol 50 μL, volume of pyridine 10 μL, volume of isobutyl chloroformate 50 μL, sonication time (1) 1 min, volume of phenylboronic acid 100 μL, concentration of phenylboronic acid 3 mg mL^{-1} and sonication time (2) 5 min.

The pH of added buffer was recognized as a crucial parameter. Derivatization of acids (GA and respective IS2) was greatly favored by addition of borate buffer of pH 9 as stated elsewhere, although adjusting of proper pH of reaction aqueous medium should be reconsidered for each single analyte during optimization [33,34]. It should be mentioned that pyridine addition is also an important parameter of derivatization procedure, as confirmed in experimental sample treatment where pyridine omission resulted in absence of GA and respective IS2 derivatives in chromatograms.

3.2. Analytical performance

Calibration curves were constructed and processed as described in section Sample pretreatment using blank human serum or urine (100 μL) spiked to 25, 50, 100, 250, 1000, 2000 and 5000 mg L^{-1} of each analyte. The coefficients of determination were in the range of 0.9980–0.9999 for analytes in serum and in the range of 0.9971–0.9991 for analytes in urine. This range covers limit for antidote treatment and severe toxic levels. The equations for the standard curves were obtained by plotting the analyte to internal standard peak area ratios against the analyte concentrations.

Limit of detection (LOD) and quantitation (LOQ) were arbitrarily set to 25 mg L^{-1} and 50 mg L^{-1}, respectively. LOD and LOQ values calculated on 3 times and 10 times response standard deviation, respectively, have proven that these values are below the selected value of 25 and 50 mg L^{-1}. This selected LOD and LOQ should be sufficient for emergency cases (see Introduction). The inter-day and intra-day precision and accuracy determined in human serum and urine at two levels of concentration of EG, PG and GA are summarized in Table 2 (urine in Table S2). The method performed well in terms of accuracy and precision over the selected concentration range, with all results being within the appropriate range of coefficient of variation 9.4% and accuracy 92.4–108.7%.

Table 2
Inter and intra-day precision and accuracy for ethylene glycol, 1,2-propylene glycol and glycolic acid in serum (four days, six replicates).

	Inter-day						Intra-day					
	Ethylene glycol		1,2-Propylene glycol		Glycolic acid		Ethylene glycol		1,2-Propylene glycol		Glycolic acid	
$c_{nominal}$ (mg mL^{-1})	50	1000	50	1000	50	1000	50	1000	50	1000	50	1000
$c_{measured}$ (mean ± SD)	48.0 ± 3	1087.0 ± 27	46.0 ± 3	1057.0 ± 27	53.0 ± 5	1087.0 ± 35	48.0 ± 4	1079.0 ± 39	46.0 ± 3	1058.0 ± 25	54.0 ± 5	1087.0 ± 33
Precision (CV %)	7.2	2.5	5.8	2.6	9.4	3.3	7.8	3.6	7.0	2.4	9.9	3.0
Accuracy (%)	96.3	108.7	92.4	105.7	106.3	108.7	95.3	107.9	91.0	105.8	107.6	108.7

Table 3
Concentrations of ethylene glycol (EG) and glycolic acid (GA) in serum from 15 patients with suspected glycol intoxication.

Patient	Admission sample (mg L^{-1})			Treatment control samples (mg L^{-1})								Treatment
	Blood pH	0		1		2		3		4		
		EG	GA	EG	GA	EG	GA	EG	GA	EG	GA	
1	7.2	1656	130	3737	111	328	0					E
2[a]	6.8	449	1177	10	376	0	135					HD
3	6.7	848	1387	76	0							HD+E
4	6.9	2335	1137	2670	1295	1641	1125	325	29	50	0	HD+E
5	6.8	768	955	115	54	26	0					HD
6	6.8	993	1317	536	334	126	0	57	0			HD
7	6.7	3582	1311	953	240	123	120	0	0			HD+E
8	7.3	2662	467	664	52	239	0	25	0	23	0	E
9	6.9	4312	435	5565	636	200	0					HD
10	7.2	308	357	190	0	0	0					HD+E
11	7.4	652	0	478	0	261	0	54	0			E
12	7.4	3079	0	1258	0	508	0	86	0	0	0	E
13	7.4	420	0	333	0							E
14	7.4	140	0	92	0	67	0					E
15[a,b]	6.9	0	630									HD

E – ethanol; HD – hemodialysis.

[a] Renal failure.
[b] Death.

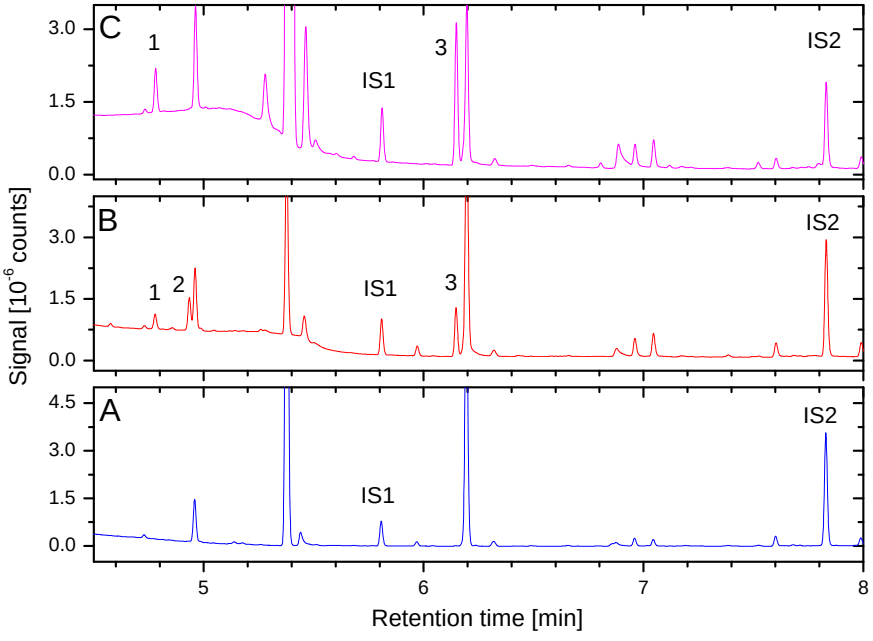

Fig. 2. Representative GC-FID chromatograms for ethylene glycol (1), 1,2-propylene glycol (2), 1,3-propylene glycol (IS1), glycolic acid (3), and 3-(4-chlorophenyl) propionic acid (IS2) standards in serum sample: (A) a blank human serum spiked with internal standards; (B) a blank human serum spiked with 250 mg mL^{-1} of all analytes; (C) a serum sample of patient n. 2. Separation conditions: Rtx-5 column (30 m × 0.25 mm ID, 0.50 μm film thickness), temperature program: 70 °C at 20 °C min^{-1} to 300 °C, total run time was 13 min, injector temperature: 250 °C, detector temperature: 310 °C, column flow: 2.82 mL min^{-1}, sample volume: 1 μL, split 1:50.

3.3. Analysis of authentic patient samples

New developed method has been used for quantitation of EG and GA in serum of 15 patients with suspected glycol intoxication (Table 3). The severity of intoxications correlates with developed metabolic acidosis due to metabolism of EG to GA, as discussed elsewhere [2,35]. Intoxication resulted in acute renal failure of patient number 2 and 15. The latter one died despite immediate medical intervention, as a result of admission in the late phase of untreated intoxication after complete metabolic conversion of EG. Patients numbered 11–14 have been taken to emergency units within one hour, with ethanol treatment being usually applied by patients themselves before admission after consultation with toxicological information center or ethanol was a component of ingested fluid. In case of poisoning with EG in combination with ethanol (combination present in some antifreeze solutions) measurement of toxic GA alone is diagnostically insufficient, because after the elimination of ethanol, significant amount of glycol can be still present in the patient's body. This may result in delayed metabolism and development of metabolic acidosis after patient release. Measurement of EG and GA in urine samples of patients may give additional information about time of ingestion and severity of intoxication (see Table S3).

Fig. 2 shows the typical GC-FID chromatograms for a blank human serum spiked with internal standards, a blank human serum spiked with 250 mg mL^{-1} of all analytes and a serum sample of patient no. 2.

4. Conclusion

In this paper, we have described the effective and fast derivatization method for simultaneous determination of EG, PG and GA in human serum and urine using GC-FID. The conditions of parallel derivatization by isobutyl chloroformate and phenylboronic acid have been optimized using experimental design. 3-(4-Chlorophenyl) propionic acid was chosen as an internal standard to avoid the possible interferences from naturally occurring endogenous acids. Presented method is a cost effective alternative to well established GC–MS methods utilizing substantial volumes of rather expensive silanization agents with minimized sample pretreatment and turnaround time about 30 min, which is required for timely effective support of a diagnosis of suspected ethylene glycol poisoning and to initiate adequate treatment. The assay was successfully applied to measurements in our routine clinical practice and the results were also presented.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.07.020.

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