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Validated gas chromatographic–mass spectrometric assay for determination of the antifreezes ethylene glycol and diethylene glycol in human plasma after microwave-assisted pivalylation ☆

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Abstract

A gas chromatographic–mass spectrometric assay is described for identification and quantification of the antifreezes ethylene glycol (EG) and diethylene glycol (DEG) in plasma for early diagnosis of a glycol intoxication. After addition of 1,3-propanediol as internal standard, the plasma sample was deproteinized by acetone and an aliquot of the supernatant was evaporated followed by microwave-assisted pivalylation. After gas chromatographic separation, the glycols were first identified by comparison of the full mass spectra with reference spectra and then quantified. The quantification has been validated according to the criteria established by the *Journal of Chromatography B*. The assay was found to be selective. The calibration curves for EG and DEG were linear from 0.1 g/l to 1.0 g/l. The limit of detection for EG and DEG was 0.01 g/l and the limit of quantification for both was 0.1 g/l. The absolute recoveries were 50 and 65% for the low quality control samples and 51 and 73% for the high quality control samples of EG and DEG, respectively. Intra- and inter-day accuracy and precision were inside the required limits. The glycols in frozen plasma samples were stable for more than 6 months. The method was successfully applied to several authentic plasma samples from patients intoxicated with glycols. It has also been suitable for analysis of EG and DEG in urine.

Keywords: Ethylene glycol; Diethylene glycol

1. Introduction

Ethylene glycol (1,2-ethanediol, EG) and diethylene glycol (2,2′-oxy-diethanol, DEG) are slightly viscous, highly hygroscopic and sweet-tasting liquids with many commercial uses, especially as antifreezes or solvents. Although toxicity of the mother substances is relatively low, accidental or suicidal ingestion of relatively high doses of EG or DEG may lead to severe intoxications [2]. In Germany, a common pediculocide contains 40% of DEG as solvent. Several life-threatening intoxications were observed following accidental ingestion. However, if the glycols can be identified and quantified in plasma during a relatively early phase of intoxication, appropriate therapeutic measures (application of ethanol or 4-methylpyrazole and/or hemodialysis) can be taken

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to prevent severe organ damage or death [2]. Furthermore, the efficiency of detoxification can be controlled by monitoring the glycol (and ethanol) plasma levels.

Several methods have been published for the determination of EG in plasma or blood using different techniques like gas chromatography (GC) with direct injection [3–6], or after isolation and derivatization using high-performance liquid chromatography (HPLC) [7], GC [8,9] or GC–mass spectrometry (MS) [10–13]. As misinterpretations of interfering peaks can lead to serious clinical or legal consequences, methods without MS detection are not sufficient [14–17]. Unfortunately, the procedures of Porter et al. [10] and of Dasgupta and co-workers [11,12] do not cover the determination of DEG. The procedure of Maurer and Kessler [13] allows additional determination of DEG. However, this procedure has some disadvantages like, e.g., rather time-consuming work-up and a sometimes rather noisy baseline. To overcome these disadvantages, we have modified this procedure and validated the new procedure according to the criteria established by the *Journal of Chromatography B* [18]. It was also tested whether this assay was suitable for glycol detection in urine.

2. Experimental

2.1. Chemicals and reagents

EG, DEG, 1,3-propanediol, pivalic anhydride and triethylamine were obtained from Fluka (Deisenberg, Germany). Methanol and acetone were obtained from Mallinckrodt Baker (Deventer, The Netherlands). All chemicals were of analytical grade or highest grade available.

2.2. Biosamples

Pooled blank human plasma samples were obtained from a local blood bank. Authentic plasma and urine samples from intoxication cases had been submitted to our laboratory for toxicological analysis.

2.3. Sample preparation

Plasma (200 μl) was mixed in a reaction vessel with 50 μl of an aqueous solution of 1,3-propanediol (1.2 g/l) as internal standard (I.S.). After addition of 1 ml of acetone, the sample was shaken for 5 min and centrifuged at 10 000 rpm. A 1-ml aliquot of supernatant was transferred to a microvial and evaporated at 65°C. The residue was dissolved in 50 μl of a freshly prepared mixture of pivalic anhydride–triethylamine–methanol (20:1:1, v/v) and incubated for 10 min under microwave irradiation at 440 W [19]. This solution was diluted with 200 μl of methanol and a 1-μl aliquot of this was injected into the GC–MS system.

2.4. Gas chromatography–mass spectrometry

2.4.1. Apparatus

The samples were analyzed using a Hewlett-Packard (HP, Waldbronn, Germany) 5890A gas chromatograph combined with an HP 5970 MSD mass spectrometer and an HP Chemstation Series G1034C-C03.00. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m×0.2 mm I.D.), crosslinked methylsilicone, 330 nm film thickness; injection port temperature, 280°C; carrier gas, helium; flow-rate, 1 ml/min; column temperature, 80°C for 6 min, then raised to 310°C at 50°C/min, final time 1 min. The MS conditions were as follows: full scan mode; electron ionization (EI) mode: ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

2.4.2. GC–MS procedures

The glycols were first indicated by mass chromatography with the ions m/z 85, 129 and 143. Positive peaks were identified by library search [20]. The identified glycols were then quantified by comparison of the peak area ratio of fragment ion m/z 85 (glycol vs. I.S.) with the calibration curve in which the peak area ratios of the standards (0.1, 0.25, 0.5, 0.75 and 1 g/l) were plotted versus their concentrations.

2.5. Assay validation for plasma analysis

The GC–MS assay was validated for the quantification of EG and DEG in plasma according to the criteria established by Lindner and Wainer [18].

2.5.1. Preparation of analytical standards, calibration standards and control samples

Standard solutions containing each the I.S. 1,3-propanediol (1.2 g/l) and EG and DEG in different concentrations (0.4, 0.8, 1.0, 2.0, 3.0, 3.2 and 4.0 g/l) were prepared in water by separate weighings. Calibration standards (0.1, 0.25, 0.5, 0.75 and 1 g/l) and quality control (QC) samples (0.2 and 0.8 g/l) of EG and DEG were prepared from the independently prepared analytical standard solutions using pooled blank plasma. For recovery studies, standard solutions containing the I.S. (0.06 g/l), EG and DEG (0.04 and 0.16 g/l each) were prepared in acetone. All solutions were stored at 4°C.

2.5.2. Peak purity and selectivity

Five different blank plasma samples were analyzed for peaks interfering with the detection of the analytes or the I.S.

2.5.3. Linearity of calibration

Calibration standards with concentrations of 0.1, 0.25, 0.5, 0.75 and 1 g/l of EG and DEG were assayed ($n=5$).

2.5.4. Repeatability

Quality control samples in the low (0.2 g/l, Low) and high concentration ranges (0.8 g/l, High) of EG and DEG were isolated and derivatized. Each sample was injected five times within a single sequence and during the course of five consecutive sequences alternately (sequence order: Low/High/Low/High/Low/High/Low/High/Low/High).

2.5.5. Accuracy and precision

Quality control samples ($n=5$) at two concentrations of EG and DEG (0.2 and 0.8 g/l each) were assayed against a calibration curve to determine the intra-day accuracy. The concentrations of the analytes were calculated by using a linear regression model and these concentrations were then compared to the nominal concentrations. The calculated values at each concentration were averaged and the percentage bias was calculated to estimate accuracy. The relative standard deviation (RSD) was calculated as a criterion of precision. The inter-day accuracy and precision of the method was assessed from the comparison of the analysis of control samples ($n=5$) on each of three consecutive days (intra-day study plus 2 additional days) in the above mentioned manner.

2.5.6. Stability

Analyte stability for long-term storage was tested by analyzing spiked samples ($n=3$) before and after storage for 6 months at −20°C. The samples were analyzed together with a freshly prepared calibration curve.

2.5.7. Limits

For determination of the limit of detection (LOD, signal-to-noise ratio greater than 3:1), quality control samples with 0.01 g/l ($n=3$) of EG and DEG were assayed. The criteria for the limit of quantification (LOQ, signal-to-noise ratio greater than 10:1) were fulfilled by the lowest point of the calibration curve (0.1 g/l EG and DEG). The data from the assay of blank matrices was taken from the selectivity experiments (cf. Section 2.5.2).

2.5.8. Recoveries

Absolute analytical recoveries were tested at the concentration levels of 0.2 or 0.8 g/l of EG and DEG ($n=5$). A solution (1 ml) containing I.S. (0.06 g/l), EG and DEG (0.04 or 0.16 g/l each) in acetone was spiked to 200 μl blank plasma and 50 μl of water. The samples were then shaken for 5 min and centrifuged at 10 000 rpm. The supernatant was transferred to a microvial and evaporated at 65°C.

As controls ($n=3$), 1 ml of the above mentioned solutions of I.S., EG and DEG in acetone was evaporated carefully at room temperature. Derivatization of the residues was carried out as described above. Recoveries were calculated by comparing the peak areas of spiked plasma samples and controls.

2.5.9. Proof of applicability

Plasma samples from authentic glycol intoxication cases were assayed with the described method.

2.5.10. Transferability of the method to urine

In order to examine, whether the method was suitable for the detection of EG and DEG in urine, transferability to the processing of urine was tested. For that purpose, spiked urine samples ($n=3$) at concentrations of 0.2 and 0.5 g/l each of EG and DEG were assayed. EG and DEG were identified via their mass spectra. Selectivity was tested by analyzing five blank urine samples.

3. Results and discussion

3.1. Sample preparation

The plasma samples were deproteinized using acetone and an aliquot of the supernatant was carefully evaporated. The residue was derivatized by pivalic acid anhydride to obtain stable glycol esters with good chromatographic properties [13]. Pivalylation has the advantage that even glycols with greater distances between the hydroxy groups (e.g., DEG) can be derivatized in contrast to the esterification by substituted boronic acids [21]. However, to ensure good derivatization results, the derivatization mixture

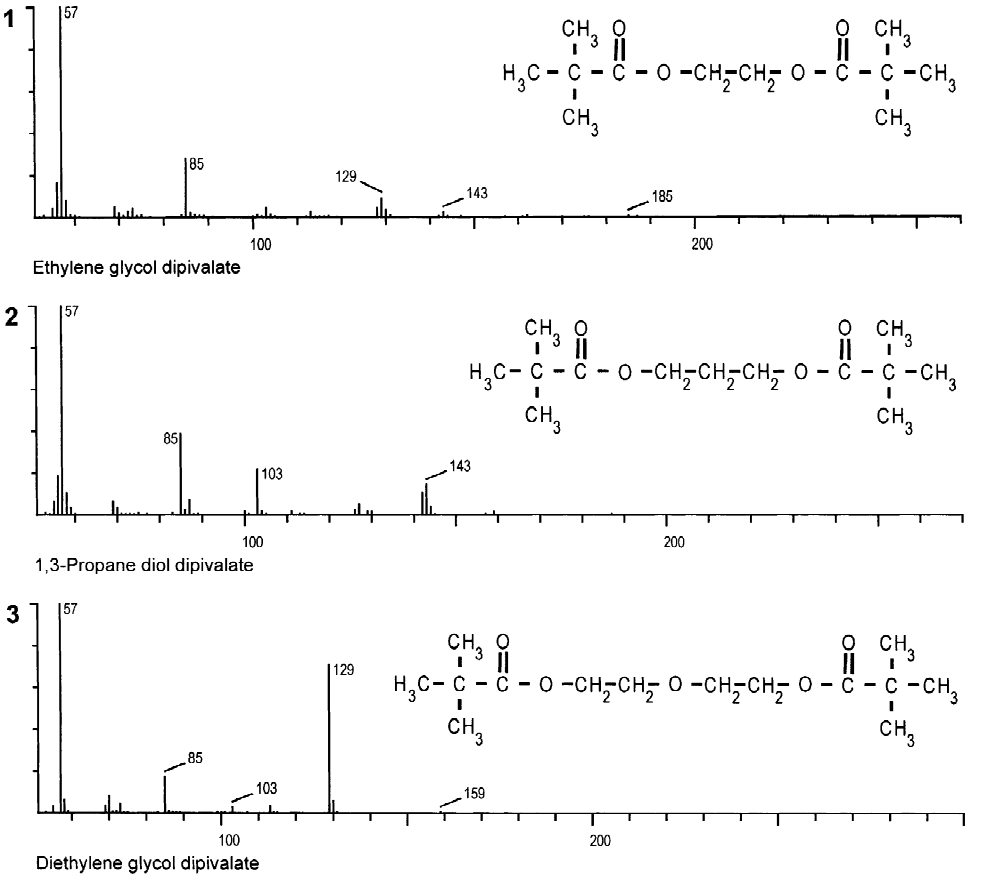

Fig. 1. EI mass spectra and structures for identification and differentiation of pivalylated EG (1), DEG (3) and the internal standard 1,3-propanediol (2). (The abscissa represents the m/z value (u), and the ordinate the relative abundances of the fragment ions (%)).

must freshly be prepared. Using microwave irradiation, our former procedure could markedly be improved. It led to higher peak abundances and better signal-to-noise ratios due to more complete derivatization and it reduced the incubation time by half [19]. 1,3-Propanediol was used as internal standard because its dipivalate has a retention time between those of the dipivalates of EG and DEG.

3.2. GC–MS identification and quantification

The glycols were first identified after isolation, pivalylation and GC separation by EI-MS. The EI mass spectra of the derivatized glycols are shown in Fig. 1 for identification via visual comparison. The mass spectra differ sufficiently so that they can be differentiated. Interference with the mass spectra of other chemicals or drugs were not observed [22,23]. In Fig. 2, merged mass chromatograms are shown of the ions m/z 85, 129, 143 of pivalylated extracts of a blank plasma sample (top) and of a blank plasma sample (bottom) spiked with 0.2 g/l each of EG (peak 1) and DEG (peak 3), and 0.3 g/l of the I.S. 1,3-propanediol (peak 2). As illustrated in Fig. 3, the mass spectra underlying the glycol peaks can also be identified by library search [20].

The identified glycol was then quantified. The peak area ratio of fragment ion m/z 85 (glycol vs. I.S.) was compared with the calibration curve in which the peak area ratios of the standards were plotted versus their concentrations.

3.3. Validation data

Mass chromatograms of a blank plasma sample are presented in Fig. 2 (top). The peak eluting before that of diethylene glycol is a matrix peak which was always sufficiently separated. In addition, only ion m/z 85 was indicated by the mass chromatograms. Therefore, confusion with DEG can be excluded.

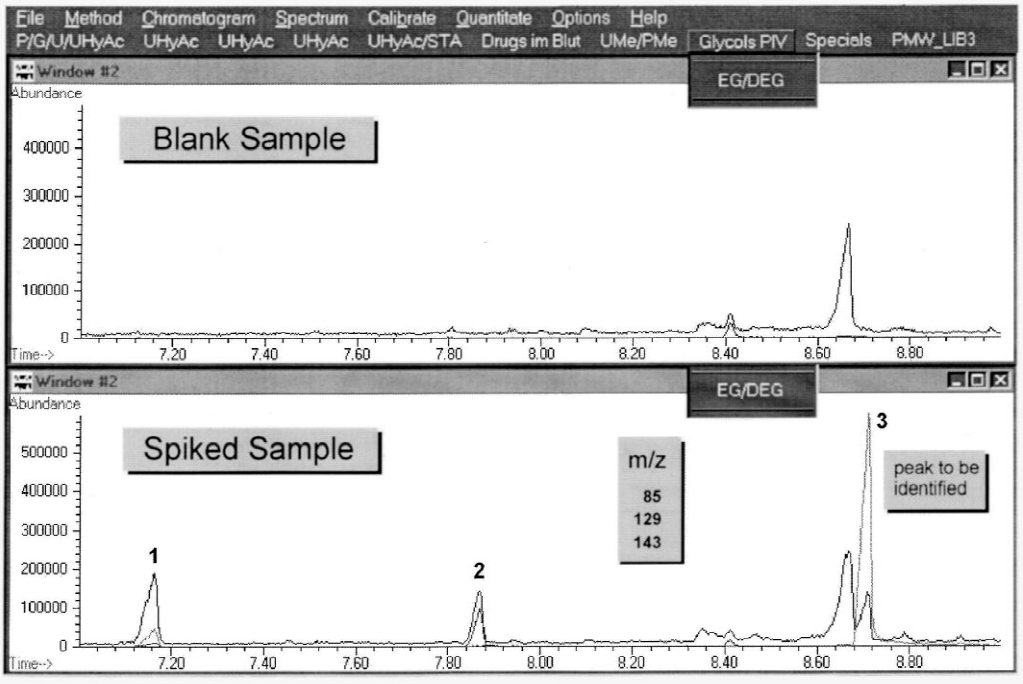

Fig. 2. Merged mass chromatograms with the ions m/z 85, 129 and 143 of pivalylated extracts of a blank plasma sample (top) and of a blank plasma sample (bottom) spiked with 0.2 g/l each of EG (1) and DEG (3), and with 0.3 g/l of 1,3-propanediol (2).

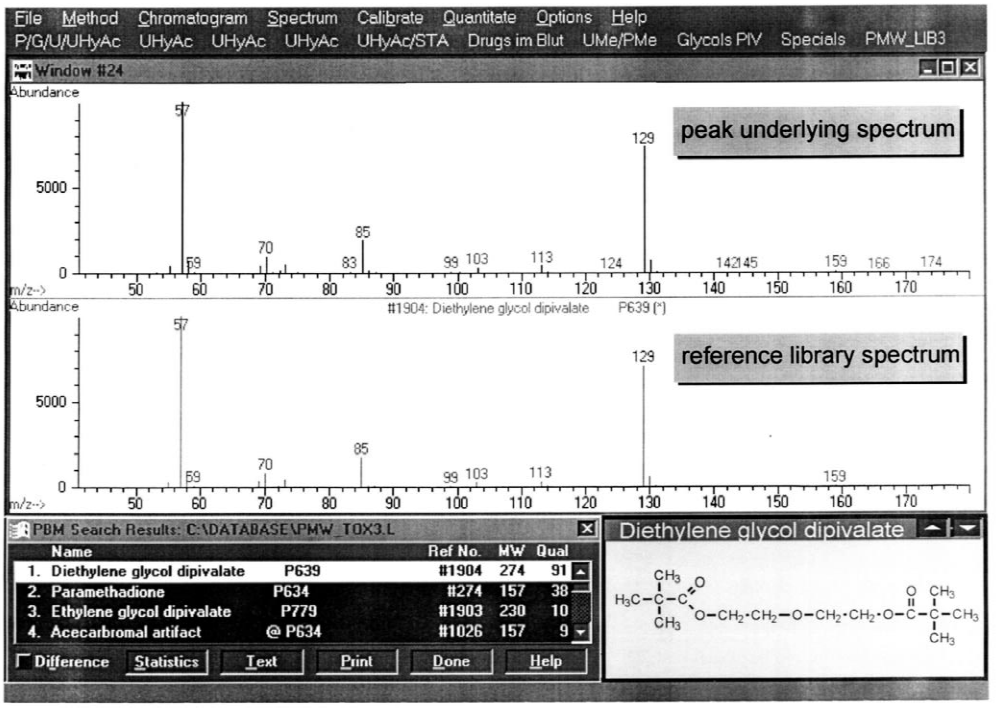

Fig. 3. Mass spectrum underlying peak 3 in Fig. 2, the reference spectrum, the structure and the hit list found by computer library search.

The calibration curve (not weighted) for EG and DEG was linear from 0.1 to 1.0 g/l with r values of 0.997 and 0.988, respectively. Repeatability was examined in the indicated manner ($n=10$, cf. Section 2.5.4). RSD values for EG and DEG were 2.9 and 3.2% for the low control samples and 3.4 and 2.9% for the high control samples. The intra- and inter-day accuracy and precision data for the quantification of the quality control samples are shown in Tables 1 and 2. The determination of spiked plasma samples before and after storage at −20°C for 6 months showed no significant differences.

The LOD was 0.01 g/l with a signal-to-noise ratio of at least 3, and the LOQ was 0.1 g/l with a signal-to-noise ratio of at least 10. This was the lowest concentration used for the calibration curve. The absolute recoveries (mean±RSD, $n=5$) of EG and DEG were 50.1±7.5 and 64.8±6%, respectively, for the low quality control samples and 50.9±12.9 and 72.6±12.1%, respectively, for the

Table 1
Intra-day accuracy and precision of the determination of EG and DEG in plasma

Intra-day ($n=5$)	Actual concentration (g/l)		Mean calculated concentration (g/l)		Precision (%)[a]		Accuracy[b]	
	EG	DEG	EG	DEG	EG	DEG	EG	DEG
Low QC	0.20	0.20	0.20	0.17	3.0	11.1	0	−15.0
High QC	0.80	0.80	0.80	0.86	6.1	8.1	0	7.5

[a] Precision=(SD/mean)·100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]·100.

Table 2
Inter-day accuracy and precision of the determination of EG and DEG in plasma

Inter-day (n=15), 3 days	Actual concentration (g/l)		Mean calculated concentration (g/l)		Precision[a] (%)		Accuracy[b] (%)	
	EG	DEG	EG	DEG	EG	DEG	EG	DEG
Low QC	0.20	0.20	0.19	0.19	9.3	11.2	−5.0	−5.0
High QC	0.80	0.80	0.81	0.82	7.2	9.9	1.3	2.5

[a] Precision=(SD/mean)·100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]·100.

high quality control samples. The recovery of the internal standard was 43.1±8.5%. The relatively low recoveries are in part due to the fact, that only 1 ml of supernatant (out of a volume of 1.25 ml) was taken for sample work-up. Another reason for the relatively low recoveries are probably evaporation losses as already discussed by Dasgupta et al. [11] and Yao and Porter [8]. This is also indicated by the better recovery of DEG, which has a higher boiling point. Nevertheless, we preferred the fast (<5 min) evaporation at elevated temperature to save time in emergency cases. Losses during evaporation were compensated by the I.S. as indicated by the good linearity, accuracy and precision. This is the first procedure for determination of EG and DEG that is fully validated according to the criteria established by the *Journal of Chromatography B* [18].

For demonstration of applicability, authentic plasma samples were analyzed. In Fig. 4 merged mass chromatograms are shown with the ions m/z 85, 129

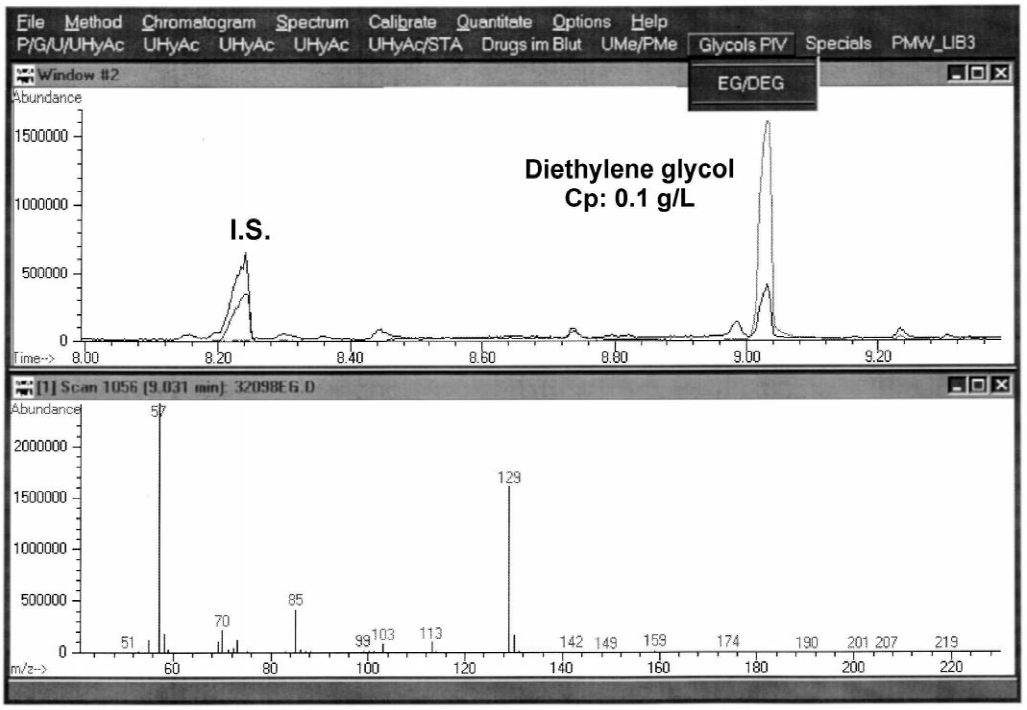

Fig. 4. Merged mass chromatograms with the ions m/z 85, 129 and 143 of a pivalylated extract of an authentic plasma sample indicating 0.1 g/l of DEG. The corresponding mass spectrum of pivalylated DEG is shown below.

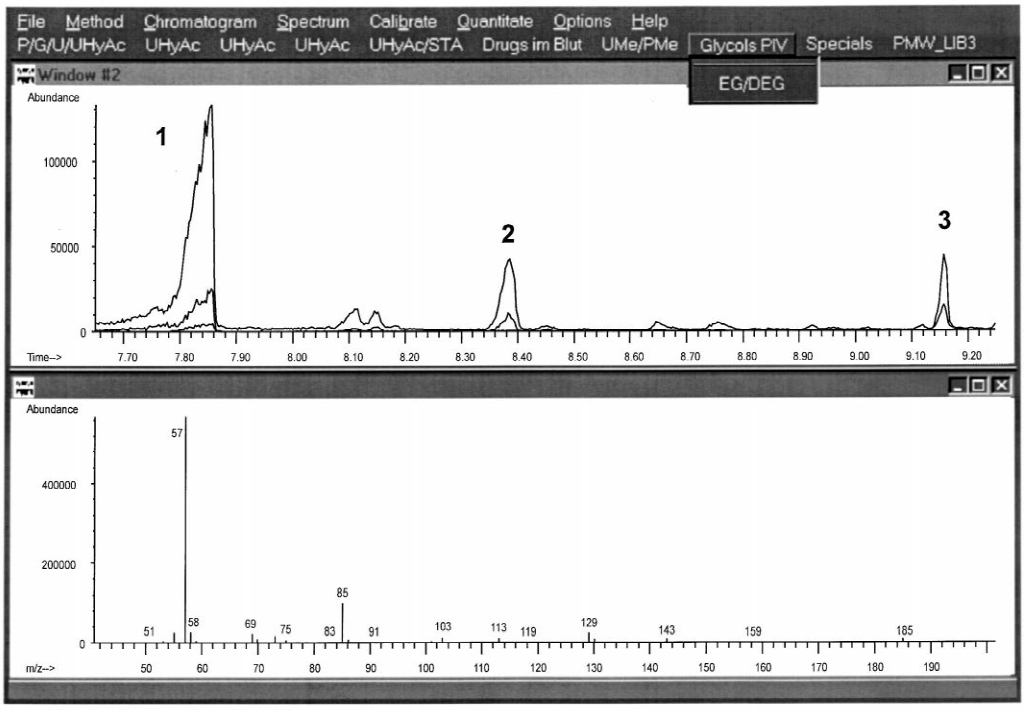

Fig. 5. Merged mass chromatograms with the ions m/z 85, 129 and 143 of a pivalylated extract of an authentic urine sample indicating EG (peak 1) and DEG (peak 3). The corresponding mass spectrum of pivalylated EG is shown below.

and 143 of a pivalylated extract of such an authentic plasma sample indicating 0.1 g/l of DEG. In the meantime, we have analyzed 33 authentic plasma samples. Eleven of them were tested positive for EG with concentrations ranging from 0.1 to 2.7 g/l. Values above the calibration range were tested after dilution with human blank plasma. Two of the samples were tested positive for DEG with concentrations of 0.1 and 0.6 g/l.

Transferability studies showed that the assay was also suitable for the detection of glycols in urine. This is demonstrated in Fig. 5, where merged mass chromatograms are shown with the ions m/z 85, 129 and 143 of a pivalylated extract of an authentic urine sample tested positive for EG (peak 1) and DEG (peak 3). In the meantime, we have analyzed 28 authentic urine samples. Eight of them were tested positive for EG and two positive for DEG.

In emergency toxicology, it might be advantageous to confine to a two-point calibration at 0.2 and 0.5 g/l to quickly assess, whether in an early EG poisoning antidote treatment (>0.2 g/l) or even hemodialysis (>0.5 g/l) is necessary [2]. This confinement should be acceptable, as this assay has proved to be linear, accurate and precise.

4. Conclusions

The GC–MS assay presented here allowed the precise and sensitive identification and quantification of EG and DEG in plasma, thus allowing the specific diagnosis of an intoxication. The method fulfilled the requirements for a validated assay. It was also suitable for analysis of urine as well as for analysis of further glycols like propylene or butylene glycols. The assay has proved to be efficient in several authentic cases.

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