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Determination of low levels of poly(ethylene glycol) 400 in plasma and urine by capillary gas chromatography–selected ion-monitoring mass spectrometry after solid-phase extraction

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Abstract

A convenient and sensitive method for the quantitative determination of poly(ethylene glycol) 400 in plasma and urine with capillary gas chromatography–mass spectrometry has been developed. The sample preparation involves solid-phase extraction with subsequent derivatization with heptafluorobutyric anhydride, which proved to give the most stable derivative. The derivatization procedure was optimized using experimental design, and different solid-phase extraction columns were evaluated. The limit of quantitation was 1 μmol/l (0.4 μg/ml) for both plasma and urine. © 1997 Elsevier Science B.V.

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1. Introduction

Poly(ethylene glycol) (PEG) is a synthetic polymer commercially available in a wide range of molecular masses from 200 to 20 000. PEG-400 is a mixture of low-molecular-mass poly(ethylene glycol)s with a mean molecular mass of approximately 400. All PEGs are water soluble and have been widely employed as food additives and in ointment bases [1]. Since they are not metabolized by intestinal bacteria and are rapidly and almost completely excreted in urine, PEGs and especially PEG-400 have been used as intestinal permeability probes [2–13]. Furthermore, PEG-400 can be used to enhance in vitro dissolution rates and bioavailability of drug substances with low solubility in water [14] and as a vehicle for intravenous administration of such compounds. In such a study on rats, one of the objects was to investigate the influence of PEG-400 on the renal function during long-term (4 days) infusion. Since the sample volumes obtained for the plasma samples were expected to be fairly small (down to 100 μl), there was a need for a sensitive and convenient method for quantifying PEG-400. In this study the concentrations of PEG in the plasma samples were 347±78 (mean±SEM) μmol/l and in urine approximately 10 times higher.

After Chadwick et al. [3,4] first developed a gas-chromatographic (GC) procedure for measuring the individual fractions of PEG-400 in biological fluids, several other analytical methods utilizing chromatographic techniques have been developed. Gas chromatography with a flame ionization detector (GC-FID) has been used for PEGs with molecular masses of 400–500 or less [3–5,9,10,14–16]. Most sample preparation procedures described for GC depend on

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lyophilization of the samples prior to extraction or on extensive sample pre-treatment with ion-exchange resins to remove interfering substances, which makes these procedures tedious and expensive in terms of time.

High-performance liquid chromatography (HPLC) has been used for the quantitation of both low-molecular- [8,11–13,17–20] and of moderate- to high-molecular-mass [6,8,11,12,17,21] polymers of PEG. The most frequent choice of detector has been the refractometer. With UV-spectrophotometric detectors, the limit of detection can be improved by introducing UV-absorbing benzoate groups [17]. Recently a new extraction procedure for HPLC analysis was introduced by Schwertner et al. [20]. They report high recoveries with a single liquid–liquid extraction using a salt–solvent combination of ammonium sulfate and dichloromethane.

In this report we describe a fast and convenient procedure for plasma and urine based on solid-phase extraction (SPE), with subsequent derivatization with heptafluorobutyric anhydride (HFBA) and selective determination by GC–MS. The method shows good recovery and reproducibility and better sensitivity than previously reported assays. The improved sensitivity enables the use of small sample volumes in future studies involving PEG-400.

2. Experimental

2.1. Chemicals and reagents

GC-grade Carbowax 400 (PEG-400) was obtained from Fluka (Buch, Switzerland) and the internal standard 1,16-hexadecanediol from Aldrich-Chemie (Steinheim, Germany). Heptafluorobutyric anhydride (HFBA) was purchased from Sigma (St. Louis, MO, USA). Solvents were of HPLC or glass-distilled grade from Rathburn (Walkerburn, UK) and other chemicals of reagent grade. Standard solutions of PEG-400 were prepared in distilled water and of the internal standard in methanol to produce working standard solutions with concentrations of 1 and 5 mmol/l, respectively. The standard solution is stable for at least 6 months when kept in a refrigerator.

2.2. Apparatus

Solid-phase extraction was performed using Chromabond C_{18} SPE columns (Macherey-Nagel, Düren, Germany) containing 500 mg of stationary phase and a SPE^{TM}-21 vacuum manifold from J.T. Baker (Phillipsburg, NJ, USA).

A Hewlett-Packard 5970 mass-selective detector coupled to an HP 5890 gas chromatograph interfaced by an open split coupling was used for the analysis. Chromatography was performed on a 10 m×0.25 mm I.D. fused-silica capillary column with an SE-54-type stationary phase (0.15 μm). The inlet pressure was 0.5 bar (helium). The column temperature was held at 110°C for 1.5 min and then programmed at 25°C/min to 300°C where it was held for 4 min. The injector was operated at 250°C in the split mode with a ratio of approximately 1:50.

The mass selective detector was operated in the selective ion monitoring (SIM) mode at m/z 241 and 436. The detector voltage was 2 kV and the open-split and connection-line temperature was 260°C.

2.3. Glassware

All glassware was washed in a laboratory dishwasher at pH 12 (Extran AP 12, Merck No. 7559), then rinsed at pH 2 (Extran AP 21, Merck No. 7559) and finally with deionized water and dried at 120°C.

2.4. Procedure

The mixed and centrifuged plasma or urine sample (0.1–1 g) was transferred to a test tube, made up to 1 g with blank plasma or urine when needed, and 50 μl of the internal standard solution was added. After mixing, the sample was transferred to the top of a C_{18} extraction column which previously had been activated with 5 ml of methanol and conditioned with 5 ml of distilled water containing 1% methanol (solvent 1).

The test tube was washed with 1 ml of solvent 1 which also was transferred to the top of the C_{18} column. An additional 4 ml of solvent 1 was then applied on the column for washing, followed by 4 ml of hexane. Finally, 10 ml of the elution solvent (15%

2-propanol in dichloromethane) was applied to the column and the eluate collected.

The eluate was evaporated to dryness under a gentle stream of dry nitrogen at room temperature. The residue was dissolved in 200 μl of toluene and 50 μl of HFBA was added. The reaction mixture was allowed to stand for 10 min at room temperature and was then evaporated to dryness under a gentle stream of dry nitrogen at room temperature. The residue was dissolved in 50 μl of toluene, and 2 μl was injected into the gas chromatograph.

2.5. Quantitation

Since PEG-400 is a mixture of poly(ethylene glycol)s with a mean molecular mass of approximately 400, the chromatogram contains a number of peaks with different intensities. Due to batch-to-batch variation, the mass distribution of these glycols may vary and it is advisable to use the same batch of PEG-400 for the standard solutions as for the experiments.

For calibration five reference samples were prepared by adding 50 μl of the PEG-400 standard solution (1 mmol/l) to 1.0 g of blank plasma. These samples were then analysed according to the procedure described above. The ratio between the peak heights of the poly(ethylene glycol) derivatives and the internal standard derivative was calculated in each chromatogram, using the highest peak from the poly(ethylene glycol)s (corresponding to a molecular mass of 326.4). The mean value of these calculated ratios was used for the estimation of the unknown sample concentrations.

During method development and routine analysis the linearity of the assay was determined by adding different amounts of PEG to blank plasma and urine corresponding to the range of concentrations covered by the method.

3. Results and discussion

3.1. Extraction

Solid-phase extraction is widely used for the clean-up and concentration of biological samples prior to chromatographic analysis [22–24] and offers, in certain instances, advantages over traditional liquid–liquid extraction techniques. The problems of finding proper immiscible solvents and tedious phase separations can be avoided. Since many SPE-columns are compatible with laboratory robot systems [25] automation is an attractive option. In many cases it is also possible to miniaturize the method by using small SPE-columns thus reducing the volumes of solvents used.

The recoveries of PEG-400 and the internal standard from a couple of SPE-columns from different manufacturers were evaluated (Table 1). The selected columns showed recoveries of 90% or more for PEG-400 but lower and more variable recovery for the internal standard. However, some of the columns gave high background of poly(ethylene glycol) which was found to originate from the two filter elements (frits), made of polyethylene, that keep the adsorbent in place in the tube. Therefore an SPE-column with frits of another material was chosen (Chromabond, Macherey-Nagel). The recovery from this column was also tested at a lower concentration

Table 1
Extraction recoveries for PEG-400 and the internal standard from plasma using different SPE columns

SPE column (trade name)	Manufacturer	PEG-400 recovery and R.S.D. (%)	Internal standard recovery and R.S.D. (%)	Poly(ethylene glycol) impurities
Bond Elut	Varian	105 (11.6)	65 (14.5)	Yes
Isolute C_{18}	International Sorbent Technology Ltd.	89 (2.4)	58 (12.3)	Yes
Isolute C_{18}-S	International Sorbent Technology Ltd.	94 (3.8)	68 (4.3)	No
Chromabond	Macherey-Nagel	93 (2.7)	82 (2.9)	No

PEG-400, 260 μmol/l; and internal standard, 300 μmol/l. The values in brackets show the relative standard deviation (n=4).

level of PEG-400 (10 μmol/l) with similar results apart from a somewhat higher relative standard deviation (12% for PEG-400 and 10% for the internal standard).

3.2. Derivatization

It is often necessary to derivatize functional groups of the analyte prior to injection into the gas chromatograph in order to improve the chromatographic performance and to enhance the detectability. For poly(ethylene glycol) the obvious choice would be either acylation or silylation to reduce the polarity of hydroxyl groups. However, emphasis was put on acylation, since earlier efforts in our laboratory with the trimethylsilylation of PEG-400 extracted from plasma were unsuccessful.

There are several acylation reagents available on the market, such as anhydrides, bisacylamides and acylhalogenides. Anhydrides are very reactive and are the classical way to produce esters of PEGs. Chadwick et al. [3,4] used acetic acid anhydride but it was later reported by Bouska and Phillips [15] that samples produced by this method appeared to be stable for only a few hours. Considering this, we compared three anhydrides: trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA). Lee [26] investigated the reactivity of these three perfluoro acid anhydrides in the formation of amides, and reported that the reactivity increased in the order HFBA< PFPA<TFAA. We could show that the stability of the derivatives increases with increasing bulkiness (Fig. 1), most probably due to the formed ester groups being sterically protected. Fig. 1 also shows that the anhydride, HFBA, used in this method produces a derivative that is sufficiently stable for analytical purposes. The derivative of the internal

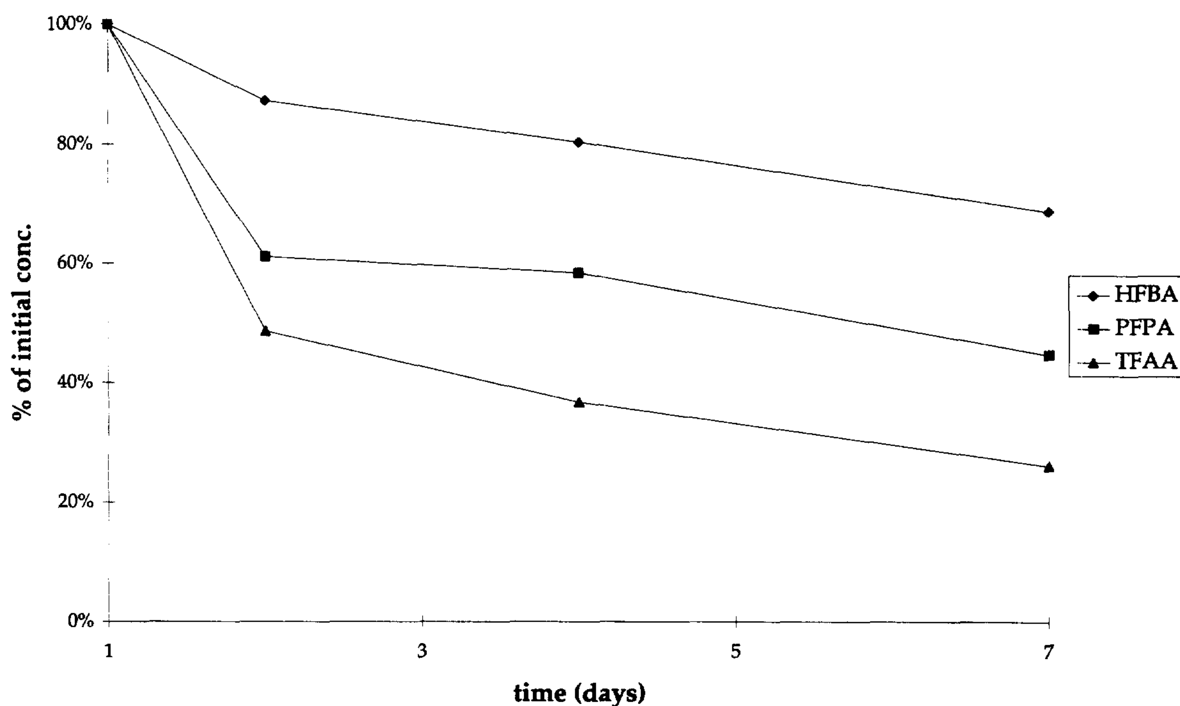

Fig. 1. Stability of three perfluoroacyl derivatives of PEG-400. The stability (in %) is measured as the peak height of the derivative relative to the original peak height day 1. The extracts were kept in a refrigerator at −20°C between analysis.

Table 2
Factors used in the factorial design

Factors	Low level	High level
Temperature (°C)	R.T.	80
Time (min)	5	60
Concentration of HFBA (%)	5 (10 μl)	33 (100 μl)

The volume of toluene was kept constant at 200 μl during the experiments.

standard was stable for several weeks. The derivatization procedure was elucidated and optimized using experimental design [27], which is a fast and convenient method to determine the appropriate experimental conditions such as time and temperature. The experiment was planned as a full factorial design with three factors; reaction time, reaction

Table 3
Worksheet showing the experimental settings and the obtained responses

Expt. no.	HFBA conc. (%)	Temp. (°C)	Time (min)	PEG-400 (rph)	Internal standard (rph)
1	5	20	5	3.768	0.320
2	33	20	5	4.163	0.294
3	5	80	5	3.776	0.320
4	33	80	5	4.187	0.312
5	5	20	60	4.002	0.288
6	33	20	60	3.774	0.313
7	5	80	60	3.755	0.297
8	33	80	60	3.900	0.342
9	20	50	33	3.735	0.337
10	20	50	33	4.217	0.328
11	20	50	33	3.941	0.321

Relative standard deviation of replicates (n=3): for PEG-400, 6.1%; for I.S., 2.4%.
Relative standard deviation of all experiments (n=11): for PEG-400, 4.8%; for I.S., 5.5%.

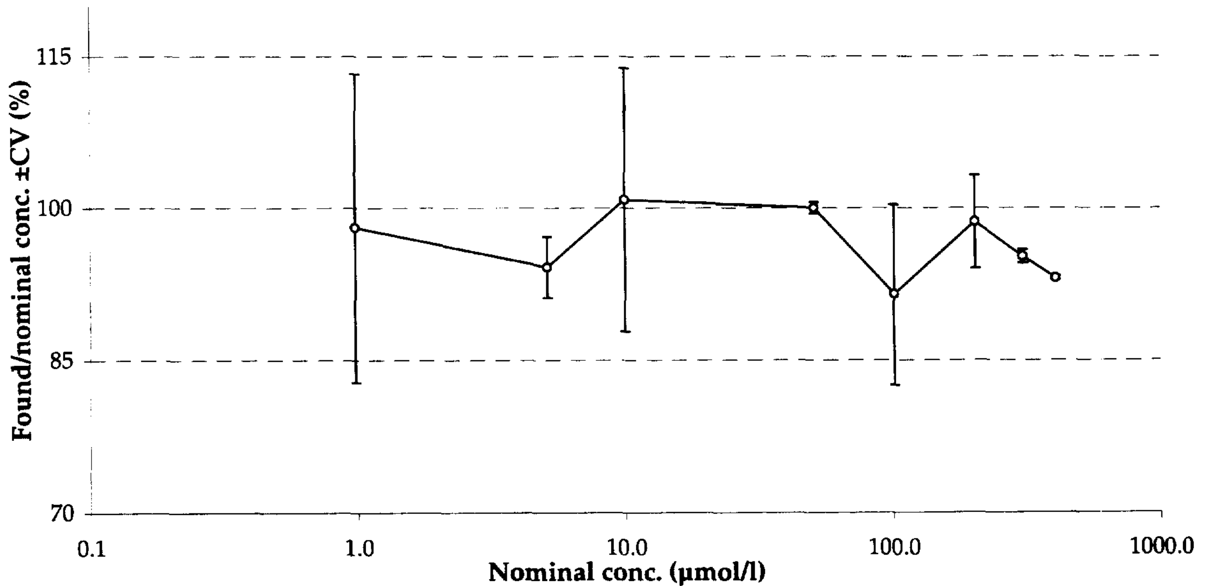

Fig. 2. Standard curve for PEG-400 in plasma. Points and bars show mean and C.V., respectively, of the ratio between found and nominal concentration at each level (n=3–6). The level 50.9 μmol/l was used for the single point calibration.

temperature and concentration of HFBA. This means that experiments were performed in such a way that all combinations of the factors at two levels were investigated (Table 2). The total number of runs was 11, including three center points. The peak heights of one of the glycols and of the internal standard were measured relative to an external standard (hexadecane) in order to increase the accuracy. The results (Table 3) showed that none of the factors actually affected the response in the selected experimental area and therefore the most economical settings were chosen for the analytical method, i.e. a fairly low concentration of HFBA (20%) and a short reaction time (10 min) at room temperature (RT). This is also a way to test the ruggedness of an analytical method.

3.3. *Quantitative evaluation*

Standard curves were constructed by analysing plasma or urine samples to which known amounts of

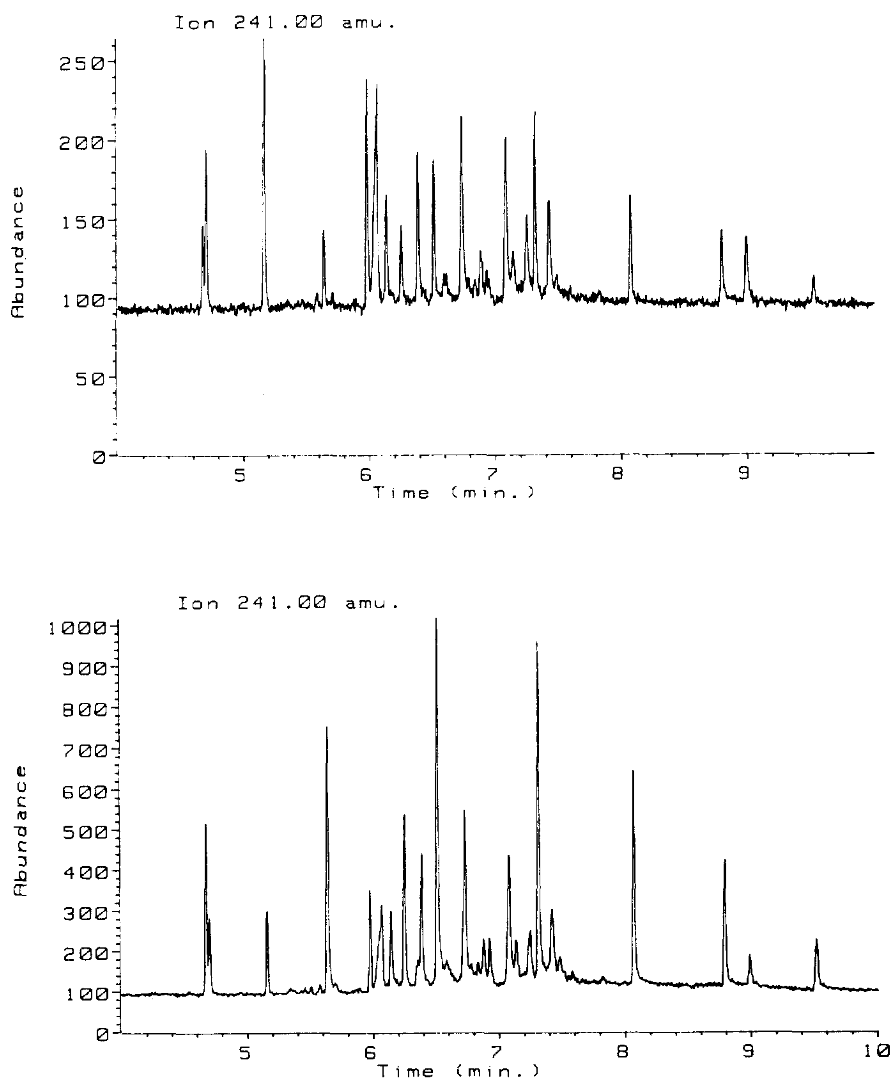

Fig. 3. Selected ion monitoring (m/z 241) chromatogram of a blank plasma sample (upper) and a spiked plasma sample containing 1 μmol/l (lower). Note that each chromatogram has been normalized.

PEG-400 had been added. In Fig. 2 a standard curve is displayed as the deviation from the theoretical line. The standard curve was linear between 1 and 500 μmol/l, where the upper concentration level is set by the risk of overloading the solid-phase extraction column. The limit of quantitation (LOQ) is defined as the lowest concentration were the standard deviation is <20% and this is valid at a concentration level of 1 μmol/l. The relative standard deviation at 50 μmol/l was 5%. Fig. 3 shows a chromatogram from a blank plasma sample and a spiked plasma sample containing 1 μmol/l. Fig. 4 shows a chromatogram from a blank urine sample and a spiked urine sample containing 1 μmol/l. The background in the blank plasma sample is higher than in the urine sample which determines the LOQ in plasma. The LOQ for urine samples can probably be considerably lowered but since the concentrations of PEG in urine were much higher we found no need to test this.

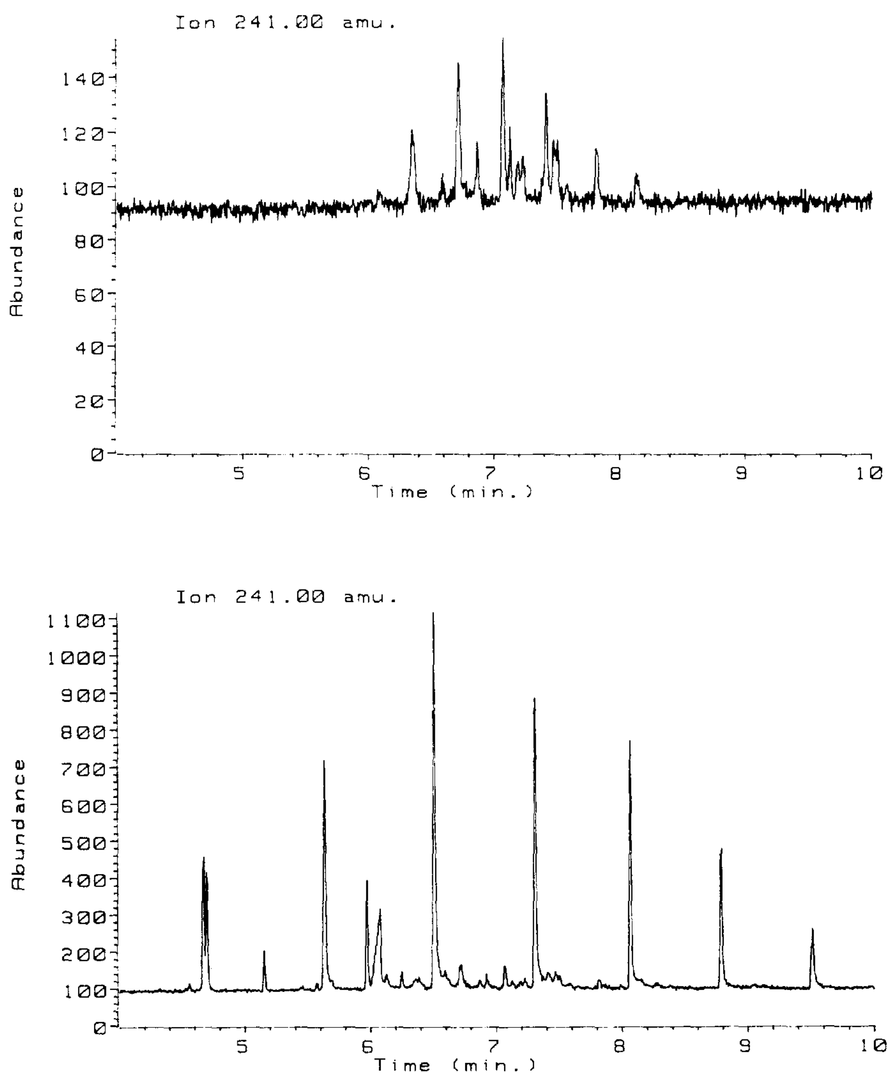

Fig. 4. Selected ion monitoring (m/z 241) chromatogram of a blank urine sample (upper) and a spiked urine sample containing 1 μmol/l (lower). Note that each chromatogram has been normalized.

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