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Clinical use of polyethylene glycols as marker substances and determination in urine by liquid chromatography

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Abstract

Adulteration of samples is a serious problem in the analysis of drugs of abuse. One of the most frequent methods is substitution of urines by "clean" urines to gain false-negative results in laboratory tests for drugs of abuse. One way to approach this problem may be to label the patient's urine with marker substances which are given orally prior to the delivery of urine. This concept is based on methods for determining malabsorption in pediatric medicine. We report a protocol for evaluating low-molecular-mass polyethylene glycols as enteral labelling marker substances. For monitoring renal excretion of the ingested polyethylene glycols we have developed and optimised an isocratic reversed-phase high-performance liquid chromatographic method with automatic sample cleanup by column switching in the back-flush technique and with RI detection. The chromatographic procedure is simple, reliable and rapid, allowing a high sample throughput for routine screening.

Keywords: Poly(ethylene glycol)

1. Introduction

In 1977, Chadwick et al. [1,2] first used polyethylene glycol (PEG) 400, composed of oligomers ranging from M_r 194 to 502, for the non-invasive in vivo investigation of intestinal permeability. Low-molecular-mass PEGs are almost completely excreted in urine with a high permeation rate. Routine separation and characterisation of PEG oligomers in clinical investigation are mainly achieved by application of high-resolution analytical techniques.

Long introduced into clinical research are gas chromatographic methods. With their demand for volatile specimens they involve extensive pre-treatment of samples besides derivatisation steps, such as, for example, acetylation [1,3,4], trimethylsilylation [5] and heptafluorobutylation [6]. Sufficiently volatile oligomers with molecular masses of less than 500 allow injection of the sample without derivatisation [7,8]. The sample cleanup procedures, however, still remain laborious. Even when volatility is enhanced by derivatisation, conventional gas chromatographic techniques are limited to PEGs with a molecular mass of up to 600, and high-temperature

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modifications are applicable for separation of molecular masses up to 800 [9], thus they do not consider the high-molecular-mass fractions of PEG 600 extending to M_r 1000.

Most frequently, PEG oligomers are separated by HPLC. Normal-phase chromatography for PEG characterisation is performed on bare silica and on the so-called bonded phases [10,11]. In clinical investigations the reversed-phase chromatographic approach is preferred because it offers the advantage of directly injecting large volumes of very dilute samples with little or no sample preparation. Routine analyses of PEGs with average molecular masses in the range 400–1000 in blood and urine are carried out mainly on C_8 [12–15] and C_{18} matrices [16,17] or on polystyrene resins cross-linked with divinylbenzene [18,19] in aqueous methanol and with refractive index detection (RID). Most procedures are quite laborious, owing to their off-line sample pre-treatment, while direct injection is used less often [15,19]. Separation of PEGs with average molecular masses of 600 and 4000 [20] and 400 and 3350 [21] in one chromatographic run without resolution into individual oligomers was achieved by gel chromatography with either aqueous [20] or organic [21] eluents and signal responses measured by means of RID.

Measuring renal PEG excretion, the widely used differential refractometry has the drawbacks of lack of sensitivity and of being limited to isocratic elution. Isocratic chromatography is not an ideal separation mode for homologous series. Advantages with respect to sensitivity can be achieved by the use of evaporative light scattering detectors, reliable and easy to handle instruments, compatible with gradients [10,11,22]. The volatility of low-molecular-mass PEGs may create problems, whereas the low-range linear response may be no handicap for characterising PEGs in urine. Detection sensitivity is one order of magnitude lower than that of UV detection [23].

The introduction of a chromophor or fluorophor into the oligomer molecules further enhances detection sensitivity. While fluorogenic labelling of PEGs is not in use in clinical laboratories, although analysis in the lower ppm range is then possible [24], pre-column derivatisation using chromophoric agents is occasionally applied. Suitable reagents are benzoyl chloride [25], benzoic anhydride [26], 3,5-dinitrobenzoyl chloride [23], phenylisocyanate [27] and naphthylisocyanate [28]. However, derivatisation reactions are time-consuming, cannot be carried out in aqueous media and are difficult to apply to volatile low-molecular-mass PEGs.

The aim of the present study was to characterise substances suitable for enteral labelling and to develop and optimise a simple and reliable HPLC method with on-line sample pre-treatment and RI detection for rapid routine monitoring of these substances in urine samples. We report optimisation experiments carried out to achieve optimum chromatographic separation conditions for appropriate resolution and maximum sensitivity and selectivity for PEG analysis in urine.

2. Experimental

2.1. Materials

PEG (Ph Eur quality) of average molecular masses 200, 300, 400, 600 and 1000 and methyl-4-hydroxybenzoate (MHB) (Ph Eur quality) were purchased from Merck (Darmstadt, Germany), HPLC-grade methanol from Mallinckrodt Baker (Gross-Gerau, Germany) and hexaethylene glycol from Sigma–Aldrich (Deisenhofen, Germany). Water was deionised and purified by Elix3 and MilliQ Gradient A10 systems from Millipore (Eschborn, Germany).

Columns: Hypersil C_8 (250 mm×4.6 mm I.D., 5 μm), Hypersil C_8 (125 mm×4.6 mm I.D., 3 μm), Inertsil C_8-3 (150 mm×4.6 mm I.D., 5 μm), Inertsil C_8-3 (250 mm×4.6 mm I.D., 5 μm), Inertsil C_{18}-3 (150 mm×4.6 mm I.D., 5 μm), Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 5 μm) and Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 3 μm), and pre-columns 60 mm×4.6 mm I.D., packed with Inertsil C_8 (5 μm), Nucleosil 100 C_{18} (5 μm) and Nucleosil 100 C_8 (5 μm) were obtained from Schambeck SFD (Bad Honnef, Germany).

2.2. Analytical equipment

The chromatographic instrumentation with an S 5200 automatic sample injector fitted with a 100 μl

injection loop, an S 2100 precolumn cleanup pump with an integrated degassing unit, a ProLab six-port motor switching valve (MSV), an RI2000-F refractive index detector of deflection type set at 30 °C and a PAT in-line filter element for PEEK 3 μm in-line filters was obtained from Schambeck SFD. An analytical M480 pump, a degasys DG1310 degassing module, a UVD 170S UV detector and Chromeleon 6.11 running under Windows NT 4.0 were purchased from Gynkotec (Germering, Germany).

2.3. HPLC analysis

Urine was centrifuged at 10 500 *g* for 10 min. The setup of the HPLC system is outlined in Fig. 1. The chromatograph was operated isocratically at ambient temperature. The eluents of both pumps were identical: 44% methanol and 56% water. Supernatants (100 μl) of the centrifuged urine were injected onto the Nucleosil 100 C_{18} pre-column (60×4.6 mm I.D., 5 μm) at a flow-rate of 0.4 ml/min. After 120 s the eluent stream was reversed by switching the pre-column to the Nucleosil 100 C_8 analytical column (125 mm×4.6 mm I.D., 5 μm). The flow-rate in the main column was 0.5 ml/min. After 240 s the system was switched back to the original status. The total analysis time was 18 min.

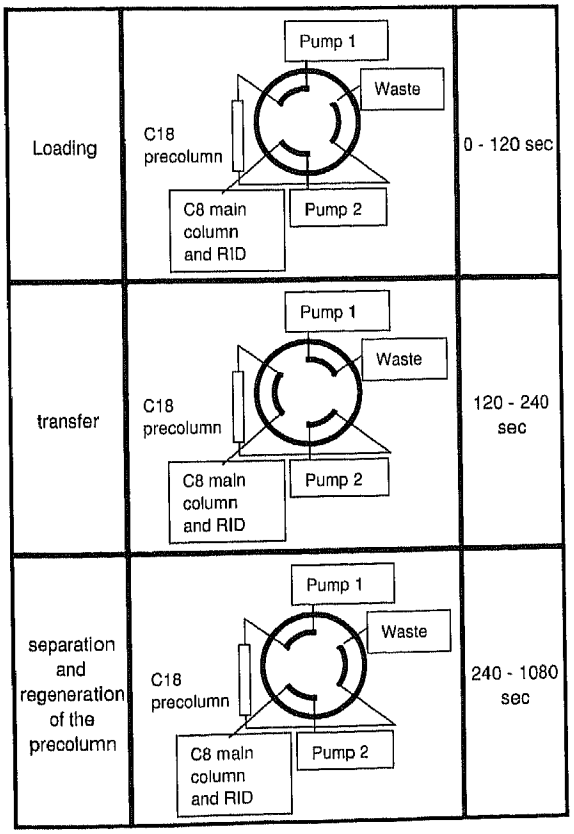

Fig. 1. Time—chart of flow—switching. For explanation, see text.

2.4. Calibration and quality control

All optimisation experiments were carried out with a PEG 600 working solution, 0.2% in urine, focussing on the oligomer with 12 repeat units. Signal assignment according to the different degrees of polymerisation was performed by spiking the PEG 600 working solution with hexaethylene glycol as a monodisperse standard.

Validation was carried out according to the European Agency for the Evaluation of Medicinal Products [29].

Stock solutions of PEG 300 and 600 (10 mg/ml each) were prepared in water and that of methyl-4-hydroxybenzoate (MHB) (10 mg/ml) in PEG 300. Working solutions (100 μg/ml each) were obtained by dilution with urine. PEG calibration curves were plotted at four concentrations (10, 20, 200 and 1000 μg/ml) by linear regression using the peak areas of the peak with a retention time (RT) of 9.7 min for PEG 300 (see Fig. 4A and D) and the peak with an RT of 14.4 min for PEG 600 (see Fig. 4B and E). MHB calibration curves were constructed by linear regression using the peak areas at five concentrations (0.01, 0.1, 1.0, 10.0 and 100 μg/ml). Accuracy and intra-assay data for 10 C.V. measurements of urines at three PEG concentrations (4, 20, and 200 μg/ml) and of MHB (0.1, 1.0 and 10.0 μg/ml) were determined. PEG-free urine was used as blank.

2.5. Specificity

Fifty randomly chosen urine samples of hospitalised patients were analysed to ensure that no endogenous PEG-like oligomeric profiles would interfere with the monitoring of the enteral labelling.

2.6. Protocol for urine delivery by volunteers

To evaluate the optimal excretion time, aqueous solutions (100 ml) of 5 g of each PEG of nominal molecular masses 200, 300, 400, 600 and 1000, either individually or in different combinations, were each drunk by one healthy volunteer. Urine was collected before and 20, 40, 60 and 120 min after administration.

A group of 20 healthy adult persons, recruited from our laboratory staff, then volunteered to drink 100 ml of water or lemon juice containing 1 ml PEG 300 or 2 ml PEG 600 or the sum of both. Urine was collected 30 min after oral administration. Intake of food or drinks was not restricted before and during the test.

3. Results and discussion

3.1. Optimisation of the chromatographic system

Optimisation experiments are outlined in Fig. 2. With respect to particle size and column length, values of plate height follow the trends predicted by theory [30], with coefficients of variation ranging from 0.53 to 7.7% for three measurements. Minimal plate height was achieved with the Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 3 μm) column with 44% aqueous methanol. This system was used for all subsequent experiments.

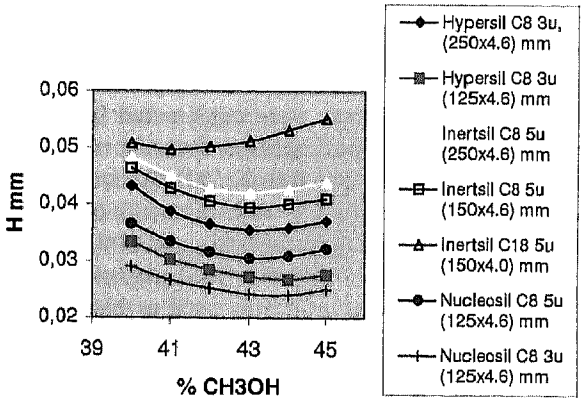

Fig. 2. Optimisation of the chromatographic system. HPLC analysis of PEG 600, 0.2% in urine, on different stationary phases with varying composition of the mobile phase. Height equivalent to a theoretical plate H as a function of % CH_3OH, performed for dodeca-ethylene glycol. Injection volume 100 μl, flow-rate 0.5 ml/min, RI detection.

3.2. Optimisation of the flow-rate

The influence of the eluent flow-rate on column efficiency is illustrated in Fig. 3. A minimum plate height was not achieved within the measured flow range. Optimal flow-rates with respect to plate height are not compatible with speed of analysis. According to the flat slope of the plate height/flow-rate profile, the loss of efficiency with increasing flow is unimportant. Therefore, instead of plate height, we selected the peak resolution R_s as the performance parameter, which yielded ideal values of 1.5 (6σ separation) at a flow-rate of 0.5 ml/min. Subsequent optimisation procedures were carried out using this flow-rate.

3.3. Optimisation of pre-column matrix and pre-column flow-rate

A pre-column was used as a protective device to separate analytes from urine compounds which cause overload of the detector. Column switching procedures were automated with a motor switching valve.

Refractivity measurement is limited to isocratic mobile phases. Therefore, the pre-column eluent is identical to that of the main column, which, in other

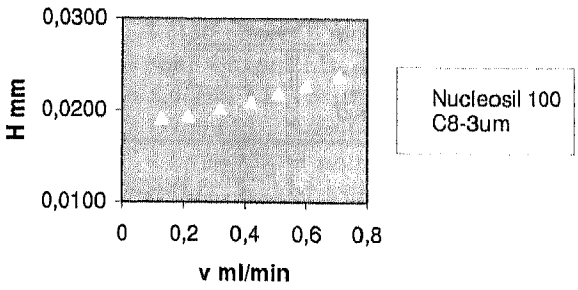

Fig. 3. Optimisation of flow-rate. HPLC analysis of PEG 600, 0.2% in urine, on Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 3 μm) with varying flow-rate. Height equivalent to a theoretical plate H as a function of the flow-rate v, performed for dodeca-ethylene glycol. 44% CH_3OH/56% H_2O, injection volume 100 μl, RI detection.

detection modes, is regarded as being contradictory to the guiding principles of pre-column cleanup. Optimisation strategy, time events and analytical results are depicted in Fig. 1 and Table 1.

Slow pre-column eluent velocities resulted in a larger number of theoretical plates for the analytical column. To increase the speed of the operation we decided to work at a pre-column flow-rate of 0.4 ml/min.

The smallest plate height was obtained in the back-flush mode using an Inertsil C_8 (5 μm) pre-column. The capacity of this precolumn is not sufficiently high for completely separating matrix interferences from the low-molecular-mass oligomers of PEG 600. Due to the fact that it was impossible to change the mobile phase, a Nucleosil C_{18} (5 μm) pre-column was chosen. With this hydrophobic material, due to the large injection volume of aqueous urine, the PEG oligomers were efficiently separated from the more hydrophilic urine impurities. The differences in H values, when compared with the optimum, are minimal. In addition, with a pre-column flow-rate of 0.4 ml/min an ideal 6σ separation is obtained for the oligomer with 12 repeat units.

3.4. Evaluation of marker substances

We intended to investigate appropriate conditions of PEG administration and urine collection to select different PEGs and excretion times suitable for enteral labelling. Therefore, we offered PEGs of different average molecular masses to one volunteer and collected urine at various excretion times.

PEG 200 is eluted within the same retention time interval as the matrix interferences and thus was not investigated further. With an eluent composition optimised for PEG 600, a combination of PEG 600 and PEG 1000 is not possible because higher M_r oligomers are more strongly retained under the experimental conditions used and therefore require gradient chromatography for their rapid elution.

PEG 400 cannot be used in combination with PEG

Table 1
Optimisation of pre-column matrix and pre-column flow-rate

Pre-column, 60×4.6 mm, 5 μm	Switching mode	v (ml/min)	RT (min)	h^a (mV)	R_s	N^b	H (mm)	MSV (s)
Inertsil C_8	Straight flush	0.3	11.7	98.2	1.89	8373	0.0149	190/380
		0.4	10.9	96.9	1.80	7250	0.0172	140/280
		0.5	10.2	94.1	1.77	6386	0.0196	110/220
	Back flush	0.3	12.5	103.9	1.70	13 603	0.0092	190/380
		0.4	11.6	97.6	1.60	10 412	0.0120	140/280
		0.5	11.1	96.0	1.52	9717	0.0129	110/220
Nucleosil C_8	Straight flush	0.3	11.7	85.6	1.96	8373	0.0149	210/240
		0.4	10.8	83.6	1.90	7170	0.0174	160/320
		0.5	10.3	84.3	1.84	6174	0.0202	130/260
	Back flush	0.3	11.9	91.8	1.57	12 656	0.0099	210/420
		0.4	10.7	89.7	1.49	10 822	0.0116	160/320
		0.5	10.5	85.6	1.44	9831	0.0127	130/260
Nucleosil C_{18}	Straight flush	0.3	11.2	87.6	1.94	9661	0.0129	180/360
		0.4	10.4	86.4	1.87	7940	0.0157	130/260
		0.5	9.9	83.2	1.81	7214	0.0173	105/210
	Back flush	0.3	11.9	89.4	1.53	11 403	0.0110	180/360
		0.4	11.1	87.9	1.50	9644	0.0130	130/260
		0.5	10.3	86.4	1.44	8331	0.0150	105/210

HPLC analysis of PEG 600, 0.2% in urine, in switching mode on different pre-column stationary phases with varying pre-column flow-rates. Analytical results for dodeca-ethylene glycol. Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 3 μm), flow-rate of analytical column eluent 0.5 ml/min, injection volume 100 μl, 44% CH_3OH/56% H_2O, RI detection.

[a] h, peak height.

[b] N, number of theoretical plates.

300 or PEG 600, because the patterns of the two individual PEGs cannot be unequivocally distinguished in the mixture due to substantial merging of oligomer signals. Even when using a gradient, the individual patterns could not be clearly recognisable [22].

PEG 300 is well separated from urine matrix constituents and presents a typical elution profile, although baseline resolution cannot be accomplished under isocratic chromatographic conditions optimised for PEG 600. Each of the three marker substances, PEG 300, 600 and 300+600, can be discriminated by its own characteristic chromatographic pattern. Chromatograms of post-ingestion urines are, therefore, easy to interpret, even by untrained personal.

The rapid renal excretion allows us to reduce the amount of PEG to ~1 g of PEG 300, 2 g of PEG 600 or the sum of both. After 20 min of oral uptake the elution patterns from urine may already be well characterised, but a minimum time period of 0.5 h should elapse to avoid problems in identification of PEGs caused by strongly diluted urines. The maximal excretion rate is attained after 1 h, and 5 to 6 h after ingestion, excretion is below the limit of RI detection.

Some patients tried to avoid the detection of illicit drugs by spitting marker substances into "clean" urine. We therefore included substances in the marker solution which would be metabolised, in contrast to polyethylene glycols. These substances should not be present in urine. One example is MHB, which we determined by UV detection at the absorption maximum of 256 nm (Fig. 4). Ten milligrams of this marker was added to 100 ml of the PEG drink solution. For this purpose a UV detector was connected on-line with the RI detector.

3.5. Calibration and quality control

Calibration of polyethylene glycol determinations, obtained by plotting the aforementioned peak areas versus urine concentrations, yielded a linear regression with $y = (0.068 \pm 0.008)x$ and $R^2 = 0.998$ for marker A and $y = (0.049 \pm 0.008)x$ and $R^2 = 0.999$ for marker B. Linearity was achieved over the concentration range 10–1000 μg/ml. Calibration of MHB determinations yielded a linear regression with $y = (10.770 \pm 0.016)x$ and $R^2 = 0.9996$. The mean values, variances, standard deviations and C.V.'s of marker A, marker B and MHB determinations at three concentrations of clinical interest are reported in Table 2.

The detection limit of MHB, defined as three times the noise signal multiplied by 3, is 0.4 μg/ml and the lower limit of quantification, defined as 10 times the noise signal, is 1.3 μg/ml.

For polyethylene glycols, the detection limit of the method is 2.4 μg/ml for marker A and 1.5 μg/ml for marker B, and the lower limit of quantification is 8.1 μg/ml for marker A and 5.3 μg/ml for marker B [29]. The method is reliable and meets the validation criteria for analytical methodology.

3.6. Specificity

Interference from urine matrix components was investigated using 50 randomly chosen urine samples from patients hospitalised for drug abuse. In one of these samples a PEG oligomeric profile was found because the patient had received macrogol (PEG 400) containing diazepam drops. All other chromatograms were almost free from coeluting interferences, showing singular small peaks not resembling PEG elution patterns.

4. Conclusion

The data presented here suggest that the determination of enteral marker substances by HPLC is a new diagnostic tool to clearly identify a urine sample as coming from a particular patient. A protocol for evaluating low-molecular-mass PEGs as marker substances suitable for enteral labelling is reported. A reversed-phase isocratic HPLC method, which can be automated, has been developed. Separation conditions have been optimised both theoretically and practically. The method is simple, easy to handle, rapid, reliable and inexpensive, and is suitable for a high throughput clinical laboratory. With its convenient on-line sample cleanup, it is more advantageous than the PEG analyses described in the literature, which require laborious, time-consuming off-line sample preparation. The pre-test and post-ingestion urines from control subjects yielded nearly complete

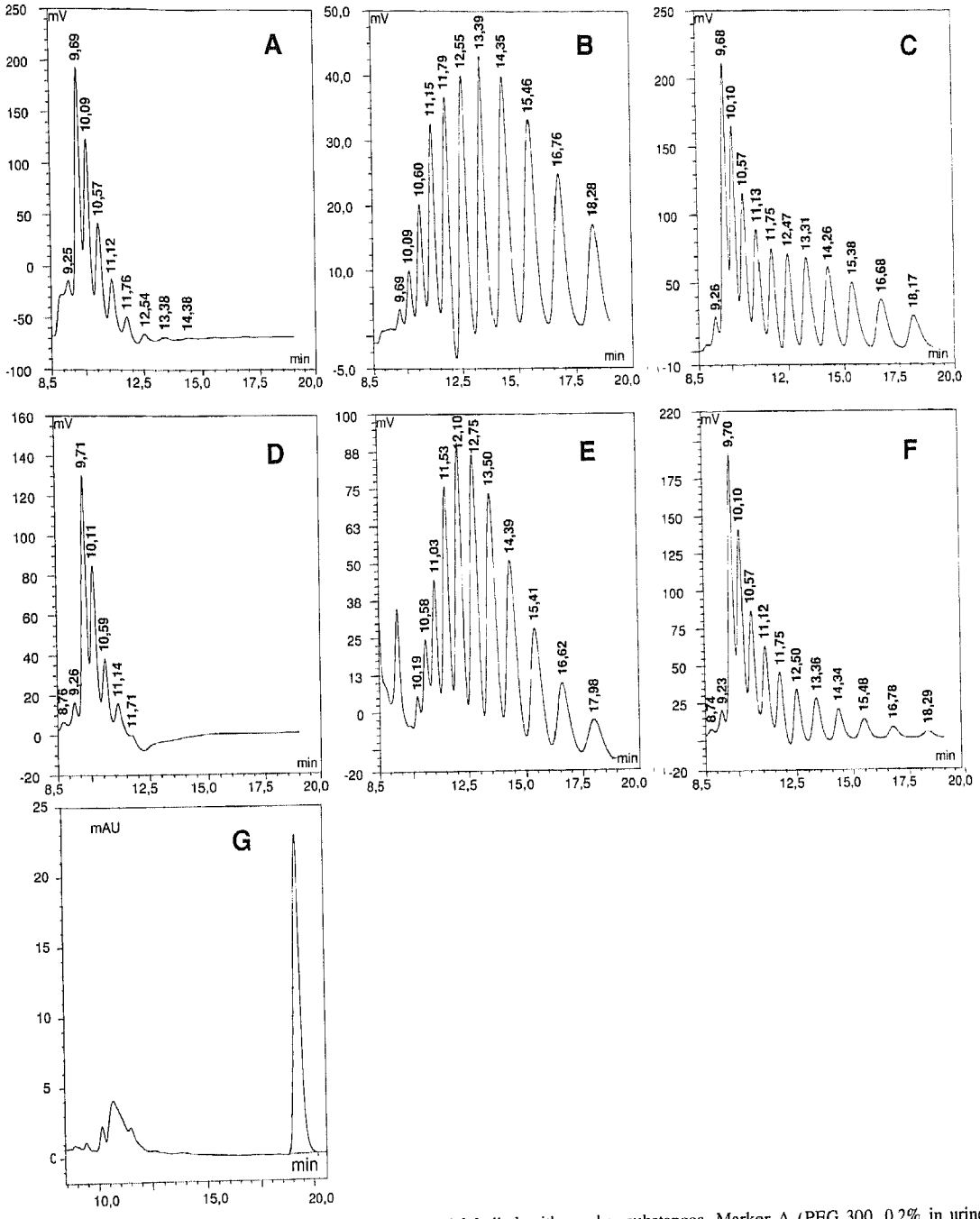

Fig. 4. Chromatographic patterns of urine samples spiked and labelled with marker substances. Marker A (PEG 300, 0.2% in urine, RI detection) (A), marker B (PEG 600, 0.2% in urine, RI detection) (B), marker C (PEG 300+PEG 600, each 0.2% in urine, RI detection) (C). Patient urines 30 min after ingestion of marker A (1 ml of PEG 300, RI detection) (D), of marker B (2 ml of PEG 600, RI detection) (E), of marker C (1 ml PEG 300+2 ml PEG 600, RI detection) (F) and 10 mg/l methyl-4-hydroxybenzoate in urine (UV detection, 254 nm) (G). Nucleosil 100 C_8 (125 mm×4.6 mm I.D., 3 μm), precolumn Nucleosil 100 C_{18} (60 mm×4.6 mm I.D., 5 μm), injection volume 100 μl, 44% CH_3OH/56% H_2O, flow column 0.5 ml/min, flow pre-column 0.4 ml/min.

Table 2
Inter-assay precision and accuracy data ($n=10$) for marker A and marker B in urine at three different concentrations

Marker A				
Concentration (μg/ml)	202.80	20.28	4.06	0 (noise)
Mean value (μg/ml)	199.56	21.30	5.05	0.81
Variance	18.79	0.25	0.58	0.11
SD	4.33	0.50	0.76	0.33
C.V. (%)	2.19	2.33	15.05	23.49
Marker B				
Concentration (μg/ml)	205.10	20.51	4.10	0 (noise)
Mean value (μg/ml)	202.39	22.21	4.04	0.53
Variance	2.95	0.23	0.56	0.25
SD	1.72	0.48	0.75	0.50
C.V. (%)	0.85	2.16	19.36	47.54
4-Hydroxy-methylbenzoate				
Concentration (μg/ml)	10.0	1.0	0.1	0 (noise)
Mean value (μg/ml)	10.26	1.02	0.12	0.13
Variance	0.02115	0.00046	0.00026	0.00402
SD	0.15	0.02	0.02	0.06
C.V. (%)	1.42	2.11	13.12	50.59

Nucleosil 100 (125 mm×4.6 mm I.D., 3 μm), pre-column Nucleosil 100 (60 mm×4.6 mm I.D., 5 μm), 44% CH_3OH/56% H_2O, flow-rate column 0.5 ml/min, flow-rate pre-column 0.4 ml/min, injection volume 100 μl, RI detection/UV detection

diagnostic sensitivity and specificity. When used with an appropriate HPLC method, low-molecular-mass PEGs appear to be ideal marker substances. A drawback of the method is the appearance of a system peak caused by switching of the motor valve. In some cases this may affect the identification of the chromatographic marker profile, in particular for very diluted urines.

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