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HPLC determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum of oncological patients after administration of morphine drugs

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A simultaneous determination of morphine (M) and its two metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), by HPLC in the serum of oncological patients is described. The compounds are extracted from the serum by means of Chromabond C_{18} – EC solid-phase-extraction cartridges, separated on a Symmetry® C_{18} analytical column (150 × 4.9 mm, 5 μm) and detected by a UV detector at 210 nm. The mobile phase consisted of 8% acetonitrile in water, 30 mmol/l phosphate buffer (pH 3) and 1 mmol/l octane sulfonic acid as the ion pairing agent; its flow-rate was 0.8 ml/min. Under these conditions, the detection limits were 10 ng/ml, 60 ng/ml and 90 ng/ml for M, M3G, and M6G, respectively. This paper concerns blood serum concentration levels of M, M3G and M6G in oncological patients, their ratios and their role in pain resistance.

1. Introduction

In 1986, morphine was recommended by the World Health Organization as the drug of choice for treatment of moderate to severe pain associated with cancer (WHO 1986).

After administration, morphine (M) undergoes extensive metabolism, which primarily occurs in the liver. Glucuronidation is the main metabolic pathway, by which morphine-3-glucuronide (M3G) predominates over morphine-6-glucuronide (M6G) production. A very small amount of normorphine is also produced. These major metabolites play a significant role in dynamic responses to morphine therapy. M6G exhibits affinity to opioid receptors similar to that of M (Mignat et al. 1995), in contrast, M3G is devoid of analgesic effects (Bartlett and Smith 1995).

Monitoring of morphine concentration and its metabolites in biological fluids is important not only for the investigation of relationships among doses of drug applied, plasma levels and analgesic effect but also for the study of the metabolic rate. The ratio of morphine concentration to the respective glucuronide concentration and the concentration ratio of both major glucuronides allows to indicate the patients who metabolize relatively more M3G than it is common and those who produce relatively more M6G metabolite.

Morphine and its metabolites have been successfully analyzed by immunoassay methods (Spector and Parker 1970; Tagliaro et al. 1989). These methods are sensitive but lack the specificity to distinguish the opiates from their glucuronide metabolites, which may cross-react with the applied antisera.

Gas chromatography (GC) (Lee and Lee 1991), alone or coupled with mass spectrometry (GC-MS) (Cone et al. 1983, Drost et al. 1984), has also been used in morphine analysis. GC separation of glucuronides has not been reported.

Isotachophoresis (ITP) (Petrovska et al. 1995) and capillary zone electrophoresis (CZE) (Wernly et al. 1993; Naidong et al. 1999) do not exhibit adequate sensitivity for biological fluids.

High performance liquid chromatography (HPLC), combined with various detection modes, is the method of choice for a simultaneous analysis of morphine and its glucuronides. Svensson's works (Svensson et al. 1982, 1986) have become the basis for many pharmacokinetic and clinical studies. The author reported an HPLC method for a simultaneous determination of M, M3G and M6G in body fluids using electrochemical detection for M and M6G, and UV detection at 210 nm for M3G. The compounds are extracted from the serum using C18 solid-phase extraction cartridges (Sep-Pak C18).

Various HPLC methods employ ultraviolet (UV) detection (Milne and Nation 1991; Chari et al. 1991; Goucke et al. 1994; Brandstetterova et al. 2002; Blahova et al. 2002; Konishi and Hashimoto 1990), fluorescence detection (Venn and Michalkiewicz 1990; Hartley et al. 1993; Huwyler et al. 1995) or electrochemical detection (ED) (Mason et al. 1991; Wright et al. 1991; Liaw et al. 1998; Wright and Smith 1998) as well as the combination of detection methods, e.g. ED and UV (Svensson et al. 1995; Ary and Rona 2001) or ED and fluorescence detection (Brandstetterova et al. 2002; Freiermuth and Plasse 1997). HPLC tandem mass spectrometric assay (HPLC-MS) (Pacifici et al. 1995; Tyrefors et al. 1996; Zuccaro et al. 1997; Schanzle et al. 1999) and HPLC-MS-MS (Zheng et al. 1998; Elsohly et al. 1988) have also been described.

A very important step in HPLC assay is the preparation of biological samples. The extraction procedures employ liquid-liquid extraction schemes (Liaw et al. 1998; Pacifici

et al. 1995; Elsohly et al. 1988; Bowie and Kirkpatrick 1989) or solid-phase extraction (SPE) techniques (e.g. Milne and Nation 1991; Wright and Smith 1998; Schanzle et al. 1999; Brandstetterova et al. 2002; Blahova et al. 2002). Great differences in the pK values of M, M3G and M6G very often cause difficulties in the SPE method development. Morphine has a ternary amine functional group with pKa of 7.9, a phenolic hydroxyl group at the C_3 carbon with pKa of 9–10, and an allyl hydroxyl group at the C_6 position. Glucuronides have a carboxylic group with pKa ranging from 3 to 4. M3G, due to glucuronidation, has no phenolic hydroxyl group (Tyrefors et al. 1996).

This paper describes an HPLC assay with UV detection for M, M3G and M6G determination in the serum of oncological patients after administration of morphine. For extracting the compounds from a biological fluid solid-phase extraction cartridges were used. Common descriptive statistics, robust statistics, and several multivariate techniques were used for the chemometrical evaluation of data.

2. Investigations and results

Forty-three patients with cancer and severe pain participated in the study. The patients had been on analgesic treatment with morphine longer than one week and the doses were not changed during the study. As analgesic preparations Slovalgin (Morphinum sulphate) or MST Continus (Morphinum sulphate) were used in different daily doses. No other opioids were given. The administration of non-opioid analgesics and other drugs (e.g. corticosteroids, antidepressants, anticonvulsants, antiemetics or sedatives) was required to maintain patient's comfort and dignity. The patients with renal dysfunction defined by a serum creatinine concentration higher than 150 μmol/l and the patients with hepatic dysfunction defined by at least two liver function tests higher than the upper limit in the hospital laboratory (20.5 μmol/l of total bilirubin, TB, 0.85 μkat/l of AST, 0.80 μkat/l of ALT, and 2.60 μkat/l of ALP) were excluded from this study.

Clinical details of the monitored patients, tumour location, treatment and daily dose of morphine are given in Table 1.

Table 1: Clinical details of patients in the study

Patient No.	Sex	Age (year)	Weight (kg)	Height (cm)	Tumour location	Treatment and doses		Daily dose (mg)
1	W*)	56	70	172	Gastrointestinal	MST*)	30 mg á 12 h	60
2	W	76	51	156	Female urogenital	MST	30 mg á 12 h	60
3	W	30	53	164	Female urogenital	Slovalgin	30 mg á 12 h	60
4	M	67	83	176	Blood tissue	MST	30 mg á 12 h	60
5	W	48	67	156	Female urogenital	MST	60 mg á 12 h	120
6	M	45	70	168	Bone	MST	60 mg á 12 h	120
7	W	63	71	168	Blood tissue	Slovalgin	60 mg á 12 h	120
8	W	50	75	164	Blood tissue	MST	30 mg á 12 h	60
9	W	46	70	178	Female urogenital	MST	60 mg á 12 h	120
10	W	47	42	158	Female urogenital	MST	60 mg á 12 h	120
11	M	21	70	185	Heart	MST	30 mg á 12 h	60
12	M	19	48	185	Blood tissue	MST	30 mg á 12 h	60
13	M	73	80	178	Gastrointestinal	MST	30 mg á 12 h	60
14	M	46	83	175	Blood tissue	MST	60 mg á 12 h	120
15	W	45	65	160	Female urogenital	Slovalgin	60 mg á 12 h	120
16	M	65	81	170	Gastrointestinal	MST	60 mg á 12 h	120
17	W	65	77	174	Female urogenital	MST	60 mg á 12 h	120
18	W	68	75	158	Female urogenital	Slovalgin	30 mg á 12 h	60
19	W	47	49	155	Lung	Slovalgin	30 mg á 12 h	60
20	M	40	76	172	Blood tissue	MST	60 mg á 12 h	120
21	M	61	64	168	Male urogenital	MST	30 mg á 8 h	90
22	M	53	95	178	Gastrointestinal	MST	60 mg á 12 h	120
23	M	39	102	187	Bone	Slovalgin	60 mg á 12 h	120
24	M	52	71	177	Lung	Slovalgin	30 mg á 8 h	90
25	W	55	41	157	Gastrointestinal	MST	30 mg á 8 h	90
26	M	66	80	173	Gastrointestinal	MST	30 mg á 12 h	60
27	W	65	69	168	Breast	MST	30 mg á 8 h	90
28	W	27	60	172	Blood tissue	Slovalgin	30 mg á 12 h	120
29	W	44	62	173	Female urogenital	Slovalgin	30 mg á 12 h	120
30	W	47	52	155	Lung	MST	60 mg á 8 h	180
31	W	47	62	158	Female urogenital	Slovalgin	30 mg á 24 h	30
32	M	65	81	170	Lung	MST	30 mg á 12 h	60
33	W	47	62	158	Female urogenital	Slovalgin	30 mg á 12 h	60
34	M	45	70	168	Bone	Slovalgin	30 mg á 12 h	60
35	M	30	70	180	Brain	MST	60 mg á 8 h	180
36	W	42	80	170	Breast	Slovalgin	30 mg á 12 h	60
37	W	37	55	155	Breast	MST	30 mg á 12 h	60
38	W	42	60	165	Female urogenital	MST	30 mg á 12 h	60
39	W	27	61	153	Female urogenital	Slovalgin	60 mg á 12 h	120
40	W	52	70	168	Female urogenital	Slovalgin	30 mg á 12 h	60
41	M	19	70	180	Bone	MST	30 mg á 12 h	60
42	M	53	85	173	Bone	MST	30 mg á 12 h	60
43	M	31	78	178	Male urogenital	MST	60 mg á 12 h	120

*) Legend: W – woman; M – man; MST – MST Continus drug

The blood for biochemical data (TB, AST, ALT, ALP) was sampled at the same time as the blood for the serum morphine and glucuronides determination.

Forty patients were satisfied with analgesic treatment but five patients made complain of pain – the patients No. 12, 32, 35, 40 and 43.

The serum concentrations of morphine and its corresponding glucuronides of all patients (found by our HPLC determination) and their ratios are given in Table 2.

It was stated in literature (Thirwell et al. 1989) that the serum concentration of morphine for a successful analgesic treatment is between 17–60 ng/ml. We have found the concentration of morphine in the blood serum samples of the monitored patients satisfied with analgesic treatment in the range 14.7–70.4 ng/ml, which is in a good agreement with the literature data. The M3G concentration in this group was 383–18180 ng/ml, and the concentration of M6G 90.8–2751 ng/ml. In the group of the patients who felt pain in spite of treatment, the concentration of serum morphine was in the range 18.8–82.1 ng/ml, the concentration of M3G in the range 2550–7711 ng/ml, and the concentration of M6G in the range 151–406 ng/ml. From these findings and further statistical evaluations it follows that there is no clear difference in the serum concentration of morphine and both glucuronides between the patients feeling pain and all others. The differences in the M3G/M and M6G/M concentration ratios between the two groups of the patients are also not distinct. However, the differences in the ratio M3G/M6G are obvious; the range is 3.07–11.1 in the group of patients without pain (the ratio exceeds 10.0 only in one case) and 10.0–21.4 in the patient group with pain. The M3G/M6G mean values and standard deviations (in brackets) are 15.2 (5.0) and 6.5 (1.9) for the patients with and without pain, respectively. The respective robust statistical values, represented by the median and MAD (the adjusted median of absolute distances from the median) are 15.0 (6.2) and 6.7 (1.7) for the same two groups. The observation of constantly higher M3G/M6G values for the group feeling pain is consistent with the results described by Tiseo et al. (1995).

Since the results of many biochemical tests are dependent of the patient's gender, the determined serum concentrations of M, M3G and M6G as well as their ratios were evaluated separately for men and women and are summarized in Table 3. Particularly informative is the ratio of the median values for men and women, respectively, which shows most pronounced differences in the c(M3G) variable and mainly in the c(M6G)/c(M) ratio; the latter is due to the fact that the men's M values are larger than the women's counterparts and the M6G values are the same or not much different. In case of the c(M3G)/c(M6G) ratio, the medians for men and women are also obviously different even though the t-test is not sufficient to prove it – its failure is connected to a very large variation in the corresponding values.

We have also studied whether the kind of administered drug – Slovalgin or MST Continus – influences the concentrations of morphine and morphine glucuronides in blood serum of the oncological patients. The corresponding results are summarized in Table 4.

Table 2: Concentration of morphine, morphine-3-glucuronide and morphine-6-glucuronide and their ratios in the serum of oncological patients

Patient No.	c(M) (ng/ml)	c(M3G) (ng/ml)	c(M6G) (ng/ml)	c(M6G)/ c(M)	c(M3G)/ c(M)	c(M3G)/ c(M6G)
1	18.8	383	90.8	4.82	20.3	4.21
2	37.4	661	99.7	2.67	17.7	6.63
3	15.6	1312	301.5	19.4	84.4	4.35
4	17.7	835	126.4	7.15	47.2	6.61
5	59.6	1825	594.8	9.98	30.6	3.07
6	44.2	2102	268.6	6.08	47.5	7.82
7	32.8	799	205.7	6.27	24.4	3.88
8	19.5	1015	312.8	16.0	52.1	3.25
9	35.8	4850	734.1	20.5	135.5	6.61
10	34.8	1044	149.5	4.30	30.0	6.98
11	32.9	3000	441.1	13.4	91.1	6.80
12	18.9	7711	405.8	21.5	408.0	19.00
13	43.9	3629	500.6	11.4	82.6	7.25
14	57.2	6214	781.4	13.6	108.6	7.95
15	47.1	2105	605.4	12.9	44.7	3.48
16	58.4	6389	1168	20.0	109.4	5.47
17	64.6	4303	1049	16.2	66.6	4.10
18	14.7	9395	290.0	19.7	63.8	3.24
19	18.5	1323	270.4	14.6	71.5	4.89
20	38.4	1254	216.2	5.63	32.7	5.80
21	49.5	3841	346.5	7.00	77.6	11.09
22	22.4	2218	312.8	14.0	99.0	7.09
23	19.5	2481	351.4	18.0	127.2	7.06
24	41.4	1851	216.2	5.22	44.7	8.56
25	37.4	2348	330.8	8.85	62.8	7.10
26	36.0	2179	345.8	9.61	60.5	6.30
27	43.8	1894	258.8	5.91	43.2	7.32
28	23.9	5505	984.5	41.2	230.3	5.59
29	15.9	3141	458.1	28.8	197.6	6.86
30	70.4	18180	2751	39.1	258.2	6.61
31	–	841	116.8	–	–	7.20
32	18.8	3227	150.8	8.01	171.4	21.40
33	37.4	661	99.7	2.67	17.7	6.63
34	17.7	946	126.4	7.15	53.5	7.48
35	82.1	7284	484.5	5.90	88.7	15.03
36	35.8	4850	734.1	20.5	135.5	6.61
37	21.8	2108	262.3	12.0	96.7	8.04
38	15.4	1459	175.1	11.4	94.8	8.33
39	32.4	2242	251.8	7.77	69.2	8.90
40	19.5	3128	312.8	16.0	160.4	10.00
41	38.1	1442	149.5	3.92	37.8	9.64
42	34.8	1452	162.8	4.67	41.7	8.92
43	65.0	2550	234.9	3.61	39.2	10.85

M – morphine; M3G – morphine-3-glucuronide; M6G – morphine-6-glucuronide. The number of digits is relevant to or by one digit larger than the corresponding precision

Table 3: Serum concentrations of M, M3G and M6G and their ratios for men, women and all patients

	c(M) (ng/ml)	c(M3G) (ng/ml)	c(M6G) (ng/ml)	c(M3G)/ c(M)	c(M6G)/ c(M)	c(M3G)/ c(M6G)
Women						
Mean	32.7	2788	477	87.3	14.9	5.99
StDev	16.1	3592	555	68.7	10.4	1.95
Median	32.8	1860	296	66.6	12.9	6.61
MAD	19.7	1437	229	53.4	9.8	2.33
Men						
Mean	38.8	3190	357	93.1	9.8	9.48
StDev	18.0	2154	256	84.6	5.5	4.41
Median	38.1	2481	313	77.6	7.2	7.82
MAD	19.7	1527	217	46.5	6.1	2.12
Men/Women						
Median	1.162	1.334	1.057	1.165	0.556	1.184
All						
Mean	35.5	2966	424	89.9	12.6	7.54
StDev	17.1	3016	448	75.4	8.8	3.67
Median	35.3	2108	302	67.9	10.7	6.98
MAD	20.5	1555	207	42.6	7.7	2.01

Table 4: Median values of the morphine (M), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) serum concentrations and their ratios with respect to their dependence on the drug administered

	Drug taken or the ratio	c(M) (ng/ml)	c(M3G) (ng/ml)	c(M6G) (ng/ml)	c(M6G)/c(M)	c(M3G)/c(M)	c(M3G)/c(M6G)
Women	MST	36.6	1860	288	57.4	10.7	6.62
Women	Slovalgin	23.9	1714	296	71.5	16.0	16.10
Women	MST/Slovalgin*)	1.53	1.09	0.97	0.80	0.67	1.09
Men	MST	38.3	2775	329	80.1	7.58	7.89
Men	Slovalgin	19.5	1851	216	53.5	7.15	7.48
Men	MST/Slovalgin*)	1.96	1.50	1.52	1.50	1.06	1.06
All	MST	37.4	2199	313	64.7	9.23	7.10
All	Slovalgin	21.7	1851	290	70.4	15.3	6.63
All	MST/Slovalgin*)	1.72	1.72	1.08	0.92	0.60	1.07

*) Dimensionless values in this row

It might also be very important to find whether tumour location has some influence on the serum concentration of morphine and morphine glucuronides, and mainly on the glucuronides ratio M3G/M6G. From the categories grouped according to tumour location only five were statistically evaluated, namely those where the number of patients was equal or larger than 4. The median is used as the measure to express the mean value in a robust way. Due to a large variability of the respective individual values in the same category, the calculated MAD values (representing the robust analogs of the standard deviation) are considerably large, which is understandable since men and women are included in the same tumour location category and there are also the patients taking different morphine drugs. Despite this, the following findings can be deduced from the results assembled in Table 5. There are no exceptional values of any variable for the F. urogenital group since they are consistent with the median values for women (Table 3). In the blood tissue group, the M3G/M6G value is lower than the median for all patients (6.98), the M, M3G median values are markedly lower and M6G/M is higher compared to the median for all patients. In the gastrointestinal group the M3G/M6G value is lower than the median value for all patients even though it should be higher with respect to the given ratio men/women. In the bone group the M3G/M6G median value is highest and comparable to the median of all men; the M3G, M6G and M3G/M medians are much lower and the M and M6G/M medians are lower than the total men's median. In the lung group the M3G/M6G as well as the M3G and M6G/M median values are higher than the median of all patients but the M and M6G median values are lower; the M3G/M value is exceptionally high.

Among the techniques of multidimensional data analysis elementary information is extracted by the principal component analysis (PCA). The goal of this method is to keep maximally the information on the data variability when the original variables are linearly combined into a lower number of principal components (PC), which are hierarchically ordered – PC1 is most informative, then PC2, PC3, etc. (Massart et al. 1997).

The PC2 vs. PC1 plot composed of four basic personal variables, dose and dose/weight ratio, as well as the "morphine" variables M, M3G and M6G (representing their respective serum concentrations) showed that the PC1 axis is a combination of the two dose and three morphine variables and the PC2 is mostly composed of weight, BMI, height and age, with a small contribution of M. The PC1 axis is therefore the "dose" or "morphine" axis, whilst the PC2 represents the basic personal data of the patients. Due to its more or less trivial content, even though well understandable, this plot is not depicted here.

More informative is the PC2 vs. PC1 plot (Fig. 1) based on the previously mentioned variables but with the ratios M3G/M, M6G/M, M3G/M6G and M6G/M3G used instead of the concentrations of M, M3G, and M6G. The

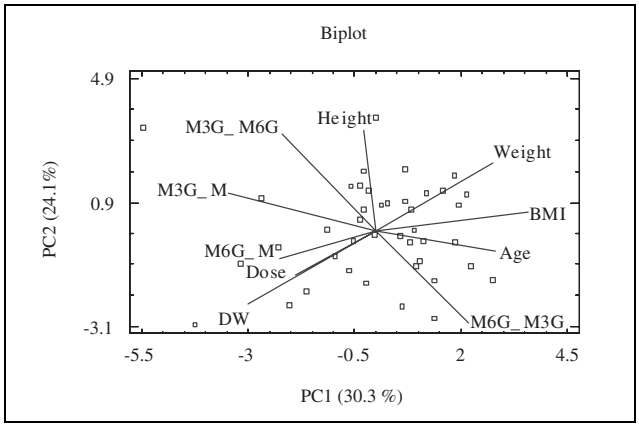

Fig. 1: Principal component analysis biplot in the coordinates of two main principal components representing the most important associations among the ratios of morphine variables, morphine daily dose, and basic personal characteristics of oncological patients

Table 5: Median serum concentration of morphine and its metabolites of oncological patients with various tumour location* (with the MAD value in brackets)

Tumour location	Number of men	Number of women	c(M) (ng/ml)	c(M3G) (ng/ml)	c(M6G) (ng/ml)	c(M6G)/c(M)	c(M3G)/c(M)	c(M3G)/c(M6G)
F. urogenital	–	14	34.8	1642	296	12.9	66.6	6.63 (2.95)
Blood tissue	4	3	23.9	1254	313	13.7	52.1	5.80 (2.84)
Gastrointest.	4	2	36.7	2284	338	10.5	72.7	6.70 (2.14)
Bone	5	–	34.8	1452	163	6.1	47.5	7.82 (1.13)
Lung	2	2	30.1	2539	243	11.3	121	7.59 (2.72)

* The results assembled in Table 3 should be used to make comparison with the median values for all 43 patients, only men (19) and only women (24), respectively

smallest angle between two rays representing the respective variables in the biplot, the strongest is the association between them; if the angle is 180° a strong but inversely proportional relationship is indicated, the 90° angle means that the variables are independent (however, all these statement are strictly valid only when the contribution of further principal components is insignificant). Thus, the opposite positions of the M3G_M6G and M6G_M3G variables, as well as weight and DW (dose/weight) are understandable and trivial, however, more meaningful is the superposition of the following pairs of variables: weight vs. dose, weight vs. M6G_M and M3G_M vs. age. From their opposite location it can be deducted that the higher doses are administered mostly to the patients with a low weight (with a more serious state), that patients with a higher weight produce a lower ratio of M6G to M serum concentrations, and that the concentration ratio M3G/M is lower for older patients. Relative closeness of height and also M3G_M to the M3G_M6G variable indicates a close relation of the mentioned variables to the M3G/M6G ratio, which is important with regard to the patient's dissatisfaction or satisfaction with the morphine treatment. The height relation to M3G_M6G is surprising and hitherto unknown but was confirmed by several independent methods (*vide infra*). One of them is Cluster Analysis, which is based on clustering variables (or objects) according to their mutual distances. The smaller the distance, the more similar are the variables (or objects) (Sharma 1996).

Fig. 2 shows clustering of 13 variables (with the single morphine variables as well as their ratios) using Ward's method and Euclidean distances. It is seen that M3G_M6G is closest to height and gender, then age and weight with BMI follow, and the largest distance in this cluster is between M3G_M6G and dose with DW.

The importance of four "personal" variables, characteristic for every individual patient, for the M3G/M6G serum concentration ratio can be quantitatively evaluated by the Canonical Correlation Analysis, by which the following equation was calculated:

$$\text{M3G\_M6G} = +1.152\,\text{height} - 0.670\,\text{weight} - 0.141\,\text{dose} - 0.129\,\text{age} \quad (1)$$

The larger the coefficient in this equation (assuming its absolute value), the more important is the respective variable for the ratio M3G/M6G, so that again height is most important, then weight is moderately important and inversely proportional to M3G/M6G. Small coefficients at dose and age indicate a low importance of these variables for the M3G/M6G ratio and the discomfort of the patients regarding pain.

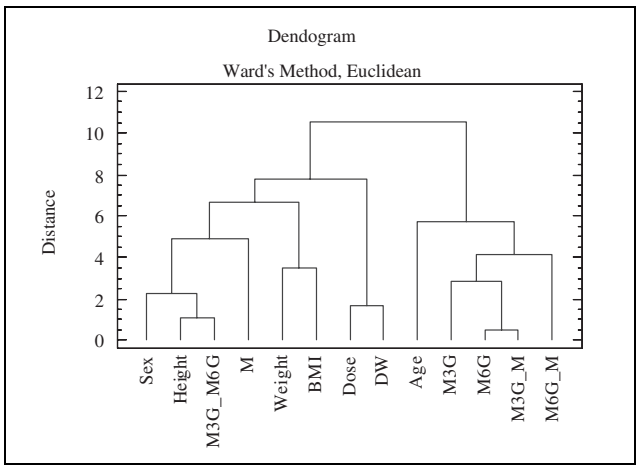

Fig. 2: Dendrogram made by Ward's method using Euclidean distances showing the distances between the given variable and any other variable. Among 13 chosen variables the basic personal characteristics of oncological patients, morphine daily dose, morphine and glucuronides serum concentrations and their ratios were used

Logistic regression (LR) (Sharma 1996) uses a categorical dependent variable, which, in our case, is M3G_M6G (the M3G/M6G ratio). For the given patient, it acquires the value of one if it is higher than the median value, and zero if it is lower or equal to the median. The independent variables can be continuous as well as categorical, so that LR is ideally suited to explore a possible effect of tumour location, represented by the corresponding categorical variable, in addition to the influence of other variables. In LR the following independent variables were used: five categorical variables of the tumour locations evaluated already in Table 5, the "personal" variables age, weight, height and dose, the morphine serum concentration (variable M), and the patient's gender (variable sex). The degree of importance of the selected variables for the M3G/M6G ratio follows from comparison of the coefficients in the logistic equation shown in the LR results output. Terms expressing the most important regression coefficients (with the absolute value larger than 1) are: 10.49^{*}bone (bone means the bone tumour location), -7.46^{*}blood tissue, -5.84^{*}sex, -5.32^{*}lung, -4.91gastrointestinal, and -3.30^{*}Furogenital. Thus, the most important variable is bone, all tumour location variables are among the best six, the only different variable in the best set is sex, all other variables seem to be of little importance by such comparison.

3. Discussion

Forty three patients undergoing analgesic treatment with morphine drugs were studied. New HPLC procedures were elaborated for the determination of the morphine, morphine-3-glucuronide, and morphine-6-glucuronide serum concentrations. Descriptive statistics, performed in a robust mode, revealed that the morphine and morphine-3-glucuronide concentrations depend on the patient's gender, with the men's values higher than the corresponding women's ones. Since the morphine-6-glucuronide serum concentration is about the same for men and women, the M3G/M6G ratio is higher for men than for women. On the contrary, the M6G/M ratio acquires larger values for women than for men.

Comparison of the data for the patients not satisfied with the morphine treatment (feeling pain) with other oncological patients clearly manifested that the higher the M3G/M6G ratio, the more patients are dissatisfied with the morphine treatment. Therefore basic statistical as well as chemometrical study of the oncological data should be oriented towards demonstration what factors influence the increase/decrease of the M3G/M6G ratio and which variables are most important from this point of view.

Several concluding remarks can be drawn when comparing MST Continus and Slovalgin as the applied morphine drugs (Table 3). The morphine serum concentrations are substantially higher both for men and women when MST is administered, which could be connected to some inactive compound (perhaps the inactive morphine stereoisomer) in the other drug. Also men's M3G and M6G levels are considerably higher in patients taking MST. For the most important M3G/M6G ratio only slightly lower values are observed for Slovalgin, both for men and wo-

men, which might be advantageous. Standard t-tests for proving the observed differences in a quantitative way have no chance to be successful owing to diversity of oncological patients; a specific role is played here by the gender of the patient, the kind of drug administered, tumour location, and perhaps further undetected factors causing that the distribution of the studied variables is not unimodal and/or not normal. Due to this fact, the robust representation of the mean value and standard deviation by the median and the MAD value is more correct.

A remarkable influence of tumour location (cancer diagnosis) on the serum concentration of morphine and its glucuronide metabolites, and consequently, on the M3G/M6G concentration ratio, was found by robust statistics (Table 5) and logistic regression. However, it would be more reliable to use a larger set of data for this purpose.

4. Experimental

4.1. Chemicals and reagents

Standard of morphine hydrochloride was obtained from the National Cancer Institute (Slovakia), M3G from Institute of Forensic Science of Slovak Police Corps (Bratislava, Slovakia), M6G from Lipomed (Switzerland). Acetonitrile and methanol (HPLC grade) were supplied by Merck (Slovakia). The following Reagent Grade chemicals (p.a.) were used: H_3PO_4, NaOH, $Na_2HPO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot H_2O$, K_2HPO_4 and $Na_2B_4O_7$ from Lachema (Brno, Czech Republic), and natrium-1-octanesulfonic acid from Pragolab (Prague, Czech Republic).

Stock standard solution of morphine hydrochloride (M) ($c = 96$ μg/ml) was prepared in methanol, stock solution of morphine-3-glucuronide (M3G) ($c = 82$ μg/ml) and morphine-6-glucuronide (M6G) ($c = 153$ μg/ml) were prepared in a mixture of methanol and water (1 : 1). All solutions were prepared diluting the stock solutions with deionised water. Linear calibrations for morphine as well as two main metabolites were made in the same way: the calibration design involved five concentrations, c, of the standard solutions of the respective compound and three replicate signal measurements, y, for each standard. The regression coefficients b_0 and b_1 and the coefficients of determination r^2 for the $y = b_0 + b_1c$ dependence of the HPLC peak area vs. concentration are listed here: $b_0 = 99.99$, $b_1 = 2.95$, $r^2 = 0.9960$ for M, $b_0 = 67.13$, $b_1 = 50.64$, $r^2 = 0.9994$ for M3G, and $b_0 = 52.94$, $b_1 = 8.41$ and $r^2 = 0.9984$ for M6G.

4.2. Apparatus

The HPLC system consisted of Delta Chrom DS 030 an HPLC pump, Watrex (Slovakia), a Rheodyne 7125 injector with a 20 μl loop, Waters Corp. (USA), an autosampler Basic-Marathon, Spark (The Netherlands) and a UV-VIS detector 484, Waters Corp. (USA). The data were collected using CSW 1 software, Microsoft (USA). The Symmetry® C_{18} (150 × 4,9 mm, 5 μm), Waters Corp. (USA) reversed-phase column was used for the HPLC separations and Chromabond C_{18}-EC cartridges (200 mg, endcup), Watrex (Slovakia), were applied for the solid-phase extraction procedure of serum samples.

4.3. Chromatographic conditions

The mobile phase consisted of 30 mmol/l phosphate buffer (adjusted to pH 3 with phosphoric acid) with 1 mmol/l octanesulfonic acid in 8% acetonitrile in water. The flow-rate of mobile phase was 0.8 ml/min at the laboratory temperature. UV detection was performed at 210 nm.

4.4. Patients and blood samples collection

Patients with cancer and severe pain were stabilized by oral controlled-release morphine tablets – either MST continus (morphine sulphate) from Mundifarma, Germany or Slovalgin Retard (morphine sulphate) from Slovakofarma, Slovakia. To minimize patient's discomfort, the blood samples, needed for this study, were taken at the same time as those for routine diagnostic biochemical tests sampled in the morning usually between 6 and 12 a.m. Venous blood samples (7 ml) were collected from a forearm vein by the service nurse using bleeding vacutainer system (vacutainer 15 027). The blood samples were centrifuged at 1200 g for 10 min and the serum was stored at −20 °C until analysis.

4.5. Extraction of biological samples

The solide-phase extraction columns Chromabond C_{18}-EC were conditioned with 10 ml of methanol, followed with 5 ml 10 mmol/l phosphate buffer (pH 2.1) containing 40% acetonitrile and 10 ml distilled water. A 1 ml aliquot of the serum sample was loaded onto the extraction column, washed with 3 ml 50 mmol/l hydrogen carbonate buffer (pH 9.3), then 20 ml 5 mmol/l hydrogen carbonate buffer (pH 9.3) was added and eluted with 1 ml of methanol. The eluate was evaporated, redissolved in 1 ml H_2O and a 20 μl aliquot was injected onto the HPLC column.

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