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DETERMINATION OF SOME BENZODIAZEPINES AND METABOLITES IN SERUM, URINE AND SALIVA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The performance of a number of liquid—solid systems, consisting of mixtures of buffers (0.05 *M*) and methanol as mobile phase and methyl-silica as stationary phase, were investigated with respect to their use in the separation of 1,4-benzodiazepines by reversed-phase high-performance liquid chromatography with UV detection at 254 nm. Phase system selectivities and column efficiencies were determined. A nomogram is presented from which the chromatographic parameters can be calculated.

A complete separation of nine benzodiazepines within 12 min has been achieved, using methyl-silica as the stationary phase and 50% methanol as the eluent.

The results were applied to the development of a method for the determination of therapeutic levels of diazepam and its metabolites in human serum, urine and saliva. The first step in the analysis, the extraction of diazepam and its metabolites from serum and urine, was also investigated and good recoveries were achieved. A low detection limit (0.2 ng) and high precision were obtained. The concentrations of diazepam and its metabolites in human serum, urine and saliva were determined after both single and multiple oral doses of diazepam (and oxazepam).

INTRODUCTION

Since the early 1960s, benzodiazepines have been widely used as minor tranquillizers, sleep inducers and muscle relaxants. A number of methods are available for determining benzodiazepines and their metabolites in body fluids, such as UV spectrophotometry, polarography [1—4] and gas [5—7] and liquid chromatography [8—11]. UV spectrophotometry and polarography are non-specific and only give information about the overall concentration of a drug and its metabolites. Gas chromatography of the intact benzo-

diazepines is possible, but the gas chromatographic analysis of the metabolites often requires their derivatization into more volatile compounds, with possible problems.

Liquid chromatography has a greater number of possibilities for adjusting the selectivity. Moreover, because of its milder working conditions, it appears to be the most suitable technique for the analysis of thermally labile, hydrophilic and hydrophobic compounds. Until now only a few papers have described the quantitative and qualitative analysis of benzodiazepines and their metabolites using high-performance liquid chromatography (HPLC). The limit of detection of these methods is often insufficient for the needs of pharmacokinetic experiments, especially at therapeutic levels. This paper reports the determination of diazepam and its metabolites in human serum, urine and saliva, after both single and multiple oral doses of diazepam (or oxazepam).

In man, diazepam is metabolized to desmethyldiazepam, oxazepam and 3-hydroxydiazepam. Diazepam is rapidly absorbed after oral administration. A single oral dose of 10—15 mg results in peak levels in serum in the range 200—300 ng/ml within 2 h. However, there is a wide variation between individuals. Chronic oral administration of 5 mg of diazepam three times daily results in steady-state serum levels of about 200—400 ng/ml.

Desmethyldiazepam is the major metabolite of diazepam in blood. At steady-state levels the concentration of desmethyldiazepam is close to or even may exceed that of diazepam. The greater part of the metabolites in urine occur as glucuronide conjugates, while only very small amounts of diazepam are present. Determination of the metabolites of diazepam in urine requires hydrolysis of the urine samples. As a consequence of the low stability of benzodiazepines under alkaline and acidic conditions, enzymatic hydrolysis by means of β-glucuronidase is often preferred.

This paper describes the separation and quantitation of benzodiazepines in serum, saliva and urine at therapeutic levels using a highly selective and efficient reversed-phase adsorption system by HPLC with UV detection.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostated glass eluent reservoir, a high-pressure pump (DMP 1515, Orlita, Giessen, G.F.R.), a flow-through Bourdon-type manometer acting as damping device, a septumless sampling device Model U6K (Waters Assoc., Milford, Mass., U.S.A.), a thermostated column (stainless-steel precision-bore tubing, I.D. 2.8 mm, O.D. 6.35 mm and length 10 cm), a single-wavelength detector (Waters 440) operating at 254 nm, a flat-bed potentiometric recorder (BD8, Kipp & Zn., Delft, The Netherlands) and a computing integrator (Spectra Physics, Autolab, System I). The damping device consisted of a flow-through Bourdon tube and a flow resistance.

Columns were packed by means of a double-headed high-pressure pump (DMP 1515, Orlita) supplied with a manometer and eight flow-through Bourdon tubes.

Chemicals and materials

Organic solvents of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and doubly distilled water were used. For the determination of very low concentrations, extraction solvents of high purity (Chrom AR Nanograde, Byk-Mallinckrodt, Wessel, G.F.R.) were used.

Buffer solution of pH 9.5 was prepared by adjusting the pH of saturated ammonium chloride solution to 9.5 with concentrated ammonia solution. The buffer solutions used in the mobile phase systems were prepared from 0.05 *M* solutions of phosphoric acid, sodium hydrogen phosphate and sodium dihydrogen phosphate. The benzodiazepines were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands) and Wyeth (Amsterdam, The Netherlands).

The methyl-silica was prepared as follows. Silica (SI 60, Merck) with a particle size range of 63—200 μm was ground in a rotating mortar. The ground materials were classified to a particle size range of 7—8 μm by means of an air classifier (Alpine MZR, Augsburg, G.F.R.). A mixture of 30 g of classified microparticulate silica, 1200 ml of toluene and 300 ml of dimethyldichlorosilane (DMCS) was refluxed with stirring for 72 h. After washing the modified silica with toluene and methanol the silica was refluxed with methanol for 6 h. The modified silica was then washed with diethyl ether and dried at 90°.

The enzyme β-D-glucuronide glucuronohydrolase (G-0251, Type B-1, Sigma, St. Louis, Mo., U.S.A.) had an activity of 10^6 Fishman units per gram of solid.

The biological samples were obtained from volunteers receiving a single dose and from patients receiving benzodiazepines therapeutically.

Column packing

Columns were packed using a slurry technique, 0.45 g of the methyl-silica was dispersed in 2 ml of carbon tetrachloride and then placed in a pre-column (I.D. 4.6 mm, O.D. 6.35 mm and length 30 cm), to which the column, closed at the bottom by a frit and a closed high-pressure valve, was attached. The pre-column was replenished with carbon tetrachloride. Then *n*-hexane was pumped into the tube, until the pressure increased to 500 bar. At this pressure, the valve at the end of the column was unscrewed. As a consequence of the change in viscosity (carbon tetrachloride 0.97 cP and *n*-hexane 0.32 cP), the pressure drop over the column decreased. After this pressure drop the pump was turned off. When the pressure had fallen completely, the column was removed and placed in the liquid chromatograph. The remaining *n*-hexane was removed by injecting three 2-ml volumes of ethanol. Then the system was ready for use.

Chromatography

The capacity ratios were calculated from the retention times of the benzodiazepines and of an unretarded compound (potassium periodate). The selectivity coefficients of a pair of compounds were calculated as the ratio of their capacity ratios. The theoretical plate height for a compound was determined from its retention time and half the peak width at 0.6 of the peak height.

Preparation of the biological samples

Urine samples. The urine was collected during 24 h. After adjusting the pH

of the urine to 5.0 with 2 *M* hydrochloric acid, a concentrated solution of 500 units of β-glucuronidase per millilitre of urine was added. After incubation at 37° for 5 h the samples were cooled to room temperature and then the benzodiazepines were extracted.

Blood samples. Blood samples of the patients receiving benzodiazepines therapeutically were taken 2—4 h after the first dose of the day. After clotting of the blood the serum was decanted and centrifuged.

Saliva samples. Saliva samples were collected from the patients immediately after taking the blood samples. The saliva was centrifuged and decanted.

Extraction

(1) Transfer 1 ml of sample, 1 ml of buffer (pH 9.5) and 1 ml of saturated potassium chloride solution [13] into a 20-ml centrifuge tube. (2) Add 10 ml of diethyl ether, mix for 60 sec (Whirlmixer), centrifuge (1000 *g* for 5 min), freeze and decant the ether phase. (3) Repeat step 2 on the aqueous phase. (4) Combine the ether phases, add 3 ml of 6 *M* hydrochloric acid, mix for 60 sec, freeze and discard the ether phase. (5) Add to the aqueous phase 1.8 ml of 10 *M* sodium hydroxide solution and 1 ml of buffer (pH 9.5), and repeat steps 2 and 3 on the aqueous phase. (6) Combine the ether phases and evaporate the solvent. (7) Dissolve the residue in 100 μl of eluent. Aliquots of 10—60 μl were analyzed by HPLC.

The determination of the higher levels does not require the complete extraction procedure. In those instances (more than 500 ng/ml), a single extraction fulfils the demands of a relatively low background. In order to achieve a rapid and efficient separation of the organic and aqueous phases after mixing, the extraction flask is placed in liquid nitrogen. The aqueous phase freezes within 50 sec and the organic phase can be decanted easily. The total extraction procedure takes less than half an hour.

RESULTS AND DISCUSSION

The choise of the phase system for a chromatographic separation should be based on the equation for the resolution, R_{ji}, for two components *j* and *i*:

$$R_{ji} = (r_{ji} - 1) \quad \frac{\kappa_i}{1 + \kappa_i} \sqrt{N_i} \tag{1}$$

where $r_{ji} = \kappa_j/\kappa_i$ = selectivity coefficient; κ_i = capacity ratio of compound *i*; N_i = number of theoretical plates for compound *i*.

In order to determine benzodiazepines and their metabolites at very low concentrations in serum, saliva and urine, the parameters of the chromatographic process should be chosen so that both the resolution and the detection limit are adequate. The relationship between the maximum outlet concentration of the solute in the mobile phase, $c_{i,m}^{\max}$, and the amount injected, Q_i, is expressed by

$$c_{i,m}^{\max} = \frac{Q_i}{\sqrt{2\pi}\epsilon_m A(1 + \kappa_i)\sqrt{H_i L}} = \frac{Q_i\sqrt{N_i}}{\sqrt{2\pi} V_m (1 + \kappa_i)} \tag{2}$$

From eqn. 2 it can be concluded that highly efficient columns and small

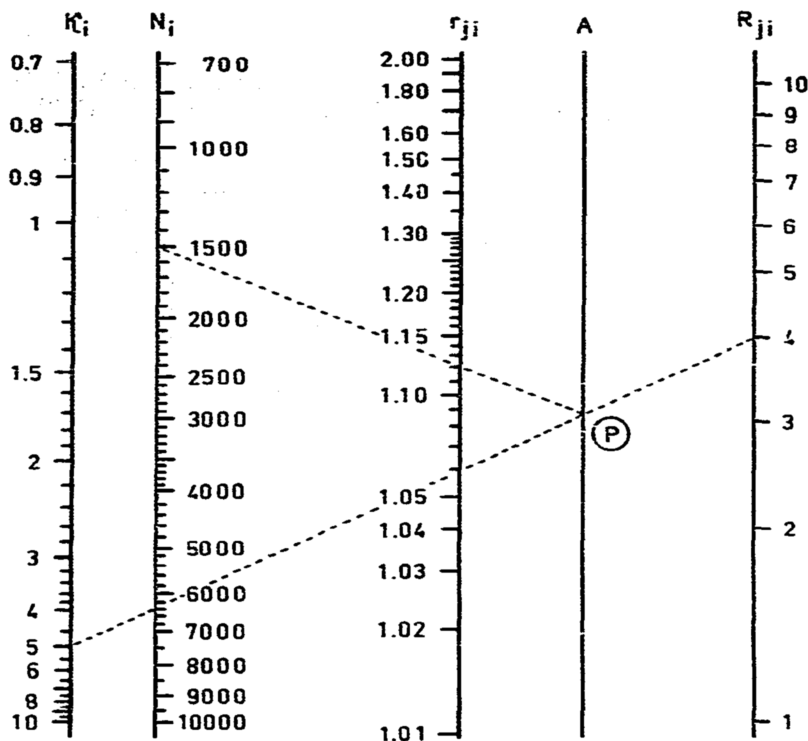

Fig. 1. Nomogram of eqn. 1.

capacity ratios are favourable for achieving low detection limits and an adequate resolution.

From eqn. 1, a nomogram can be constructed (Fig. 1). The selectivity coefficient and hence the capacity ratio of the subsequent compound can be found if the other parameters are known. In the same way, the theoretical plate number for a given phase system or the resolution can be obtained if the other parameters are known.

The nomogram is used as follows. Combining eqns. 1 and 2 it can be concluded that for minimum peak broadening and therefore a maximum outlet concentration, the resolution must be as low as practicable. In order to quantitate two components, i and j, the resolution must be equal to at least 4. The line connecting $\kappa_i = 5$ and $R_{ji} = 4$ intersects the auxiliary line A at point P. If 1500 theoretical plates are available, it can be found that the selectivity coefficient, r_{ji}, is 1.12 or $\kappa_j = 5.60$.

Similarly, if the capacity ratios and therefore the selectivity coefficient and the plate number are known, the resolution can be found easily. The line drawn through $N = 1500$ and $r_{ji} = 1.12$ intersects the line A at point P. This means that if $\kappa_i = 5$ the resolution will be 4.

Phase system selectivity

The capacity ratios and selectivity coefficients of successively eluted benzodiazepines were measured as a function of the pH of the mobile phase. The results are given in Table I.

TABLE I

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF A NUMBER OF BENZODIAZEPINES AS A FUNCTION OF THE pH* OF THE MOBILE PHASE (METHANOL–WATER, 40:60)

Component	pH = 2.4		pH = 4.2		pH = 5.9		pH = 7.0	
	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}
7-Aminonitrazepam	0.00	—	1.04	—	1.10	—	1.54	—
Bromazepam	1.77	—	3.69	3.55	3.17	2.88	4.15	2.69
Nitrazepam	2.93	1.66	5.58	1.51	4.54	1.43	5.85	1.41
Oxazepam	6.75	2.30	7.22	1.29	5.63	1.24	7.32	1.25
Nor-3-hydroxyflurazepam	6.99	1.04	6.91	0.96	4.45	0.79	7.34	1.07
Clonazepam	7.33	1.05	7.58	1.10	6.39	1.44	7.97	1.02
Norchlordiazepoxide	0.86	0.12	3.41	0.45	5.95	0.93	8.09	1.02
Lorazepam	8.88	10.3	8.69	2.55	7.09	1.19	9.27	1.15
Hydroxydiazepam	9.94	1.12	10.5	1.21	8.01	1.13	10.6	1.14
Chlordiazepoxide	0.94	0.09	5.74	0.55	8.82	1.10	11.9	1.12
Desmethyldiazepam	2.90	3.09	4.84	0.84	12.0	1.36	13.8	1.16
Norflurazepam	9.00	3.10	12.7	2.62	10.1	0.84	14.1	1.02
Flurazepam	2.24	0.25	3.08	0.24	7.73	0.77	15.2	1.08
Diazepam	11.0	4.91	20.1	6.53	18.1	2.34	20.7	1.36
Prazepam	30.0	2.73	62.6	3.11	52.0	2.87	62.2	3.00
Medazepam	2.40	0.08	5.82	0.09	45.2	0.87	91.2	1.47

*pH measured in buffer solution.

If other conditions remain the same, the capacity ratios decrease with increasing methanol content and the selectivity coefficients decrease regularly with increasing methanol content. Hence there is no difference in the order of elution. However, on changing the pH of the mobile phase, it can be seen that both the selectivity coefficients and the capacity ratios are affected.

In Fig. 2 the plot of log κ versus pH is given for a number of benzodiazepines. Between pH 4 and 6 the traces intersect and, as a consequence, the order of elution will be inverted.

In order to detect very low amounts of, e.g., desmethyldiazepam, one has to use buffer of low pH. Because of the dependence of the maximum outlet concentration on the capacity factor, it is preferable to elute first those components which are present at the lowest concentration. In order to adjust the capacity factors to more appropriate values (e.g., $\kappa > 2$), the methanol content of the mobile phase can be decreased.

Fig. 3 shows the separation of a test mixture of benzodiazepines, showing the excellent selectivity of the phase system. It can be concluded that the phase system can be applied for qualitative determinations. In this work, the determination of diazepam and its metabolites in particular has been investigated.

Composition of the extraction solvent

The extraction of the benzodiazepines from the biological matrix is an important step in this type of trace analysis, on the one hand to remove interfering substances and on the other to enrich the benzodiazepines. Benzodiazepines are weakly basic compounds with pK_a values ranging from 1.7 to 12.0.

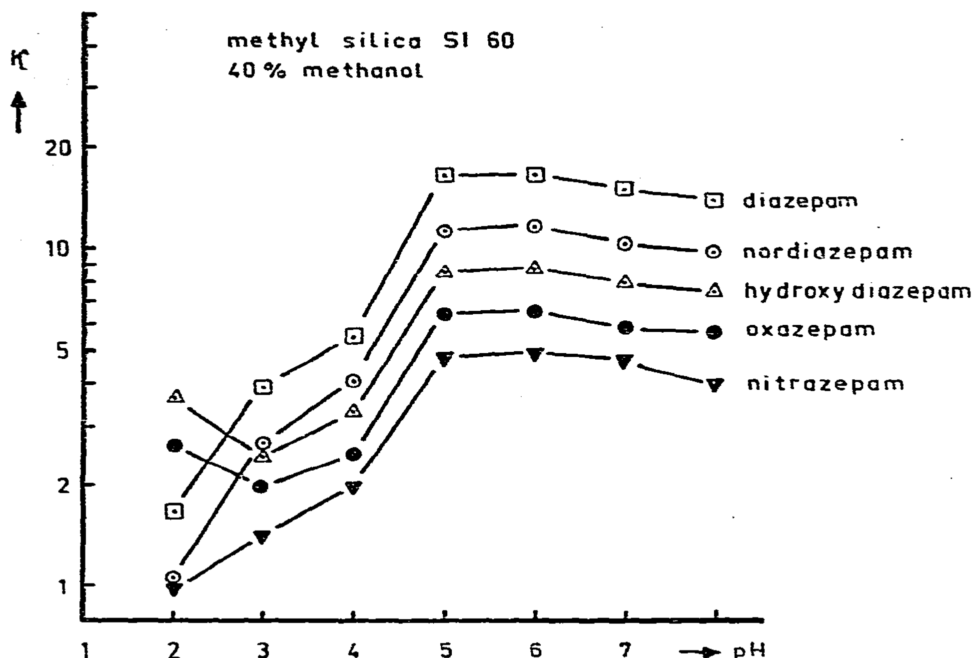

Fig. 2. Plot of the logarithm of the capacity ratio versus the pH of the mobile phase (40% methanol).

The distribution of these basic compounds between an organic solvent and an aqueous solution depends on the pH of the aqueous phase and on the pK_a value of the benzodiazepines. The total distribution coefficient of a base B, defined as the ratio of the concentrations in the organic and the aqueous phases, excluding side reactions, can be expressed by

$$K_B = \frac{[B]_{org}}{[B]_{aq} + [HB^+]_{aq}} = \frac{[B]_{org}}{[B]_{aq}} \cdot \frac{1}{1 + \frac{[H^+]}{K_a}} \tag{3}$$

where the subscripts org and aq refer to the organic and aqueous phases, respectively.

The composition of the extraction solvent determines the value of K_B. From eqn. 3 it can be seen that pH>>pK_a is favourable for obtaining high distribution coefficients, i.e., high recoveries. Many data are available in the literature on the effect of the composition of the organic phase on the distribution of the benzodiazepines. However, many of those data were obtained with extraction solvents containing highly UV-absorbing constituents (e.g., toluene or benzene). The use of this kind of solvent adversely affects determinations at the nanogram level unless the organic phase has been evaporated completely.

A number of extraction solvents were investigated. Table II gives recoveries for diazepam extracted from an aqueous solution (1 µg/ml).

The highest recoveries were obtained with diethyl ether, the addition of *n*-hexane decreased the recoveries and increased emulsification.

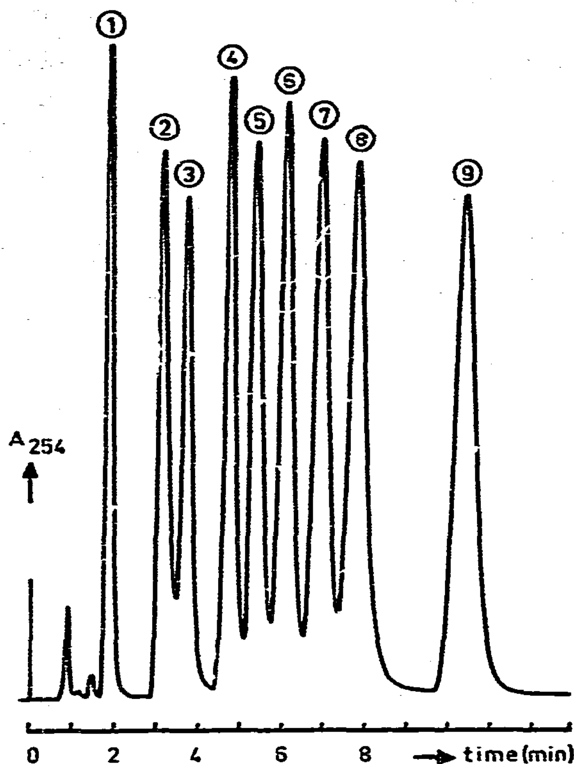

Fig. 3. HPLC separation of a test mixture of nine benzodiazepines. Column: 10 cm × 2.8 mm I.D., methyl-silica 60. Eluent: methanol—phosphate buffer (0.05 *M*, pH 6.0) (1:1). Flow-rate: 9.3 µl/sec. Peaks: 1 = 7-aminonitrazepam, 2 = bromazepam; 3 = nitrazepam; 4 = oxazepam; 5 = desmethylchlordiazepoxide; 6 = hydroxydiazepam; 7 = chlordiazepoxide; 8 = desmethyldiazepam; 9 = diazepam.

Table III gives recoveries of diazepam and its metabolites extracted from water, urine and serum, with coefficients of variation in the range from 20 to 1000 ng/ml in parentheses. The recoveries given in Table III were obtained after addition of 1 ml of saturated potassium chloride solution in the first

TABLE II

RECOVERIES OF DIAZEPAM FROM AN AQUEOUS SOLUTION (1 µg/ml) EXTRACTED WITH DIFFERENT SOLVENTS

Extraction solvent (%, v/v)			Recovery diazepam (%)
n-Hexane	Diethyl ether	*n*-Propanol	
100	—	—	52.0
50	50	—	70.1
49	49	2	68.1
48	49	3	69.9
40	60	—	79.6
—	100	—	88.3

extraction step, otherwise lower recoveries were obtained (e.g., 16% loss for desmethyldiazepam).

Preparation of urine samples

The urinary metabolites of diazepam, i.e., oxazepam, desmethyldiazepam and 3-hydroxydiazepam, are present mainly as glucuronide conjugates. As a consequence of the high polarity of these conjugates, the assay of the metabolites of diazepam in urine requires either a completely different phase system and a modified extraction procedure or an enzymatic hydrolysis. Because of difficulties in the extraction of the conjugates we chose to hydrolyse them into the free benzodiazepines. The optimal conditions for the hydrolysis were found to be pH 5.0 and 37°.

The hydrolysis is complete after 5 h; a longer period of hydrolysis results in lower recoveries, owing to the low stability of the compounds of interest.

Precision and linearity of the method

The linearity and precision of the quantitative determination of benzodiazepines by HPLC were investigated by injecting different amounts of benzodiazepines. Table III includes the coefficients of variation for test mixtures of diazepam and its metabolites. The coefficients of variation range from

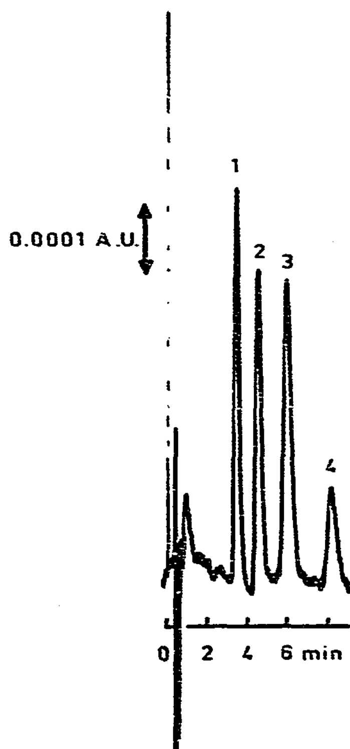

Fig. 4. HPLC separation of a test mixture of 1 ng of diazepam and 2 ng of its metabolites. Column as in Fig. 3. Eluent: methanol—phosphate buffer (0.05 *M*, pH = 7.0) (2:3). Flow-rate: 20.3 µl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam; 4 = diazepam.

TABLE III

RECOVERIES AND COEFFICIENTS OF VARIATION (C.V.) OF DIAZEPAM AND ITS METABOLITES FROM WATER, URINE AND SERUM

Component	Test mixture (20–1000 ng/ml): C.V. (%)	Mean recoveries and coefficients of variation					
		water (40–2000 ng/ml):		urine (40–2000 ng/ml):		serum (40–2000 ng/ml)	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Oxazepam	0.5–0.2	91.4	3.5–0.7	88.8	4.0–0.7	86.3	4.2–1.7
Hydroxydiazepam	1.0–0.3	91.7	3.4–1.0	90.4	4.3–1.0	88.6	4.0–1.6
Desmethyldiazepam	1.2–0.4	92.1	3.4–1.3	91.2	3.9–1.4	89.4	4.2–1.6
Diazepam	1.3–0.5	94.8	3.3–1.2	92.0	3.5–1.4	90.3	4.3–1.9

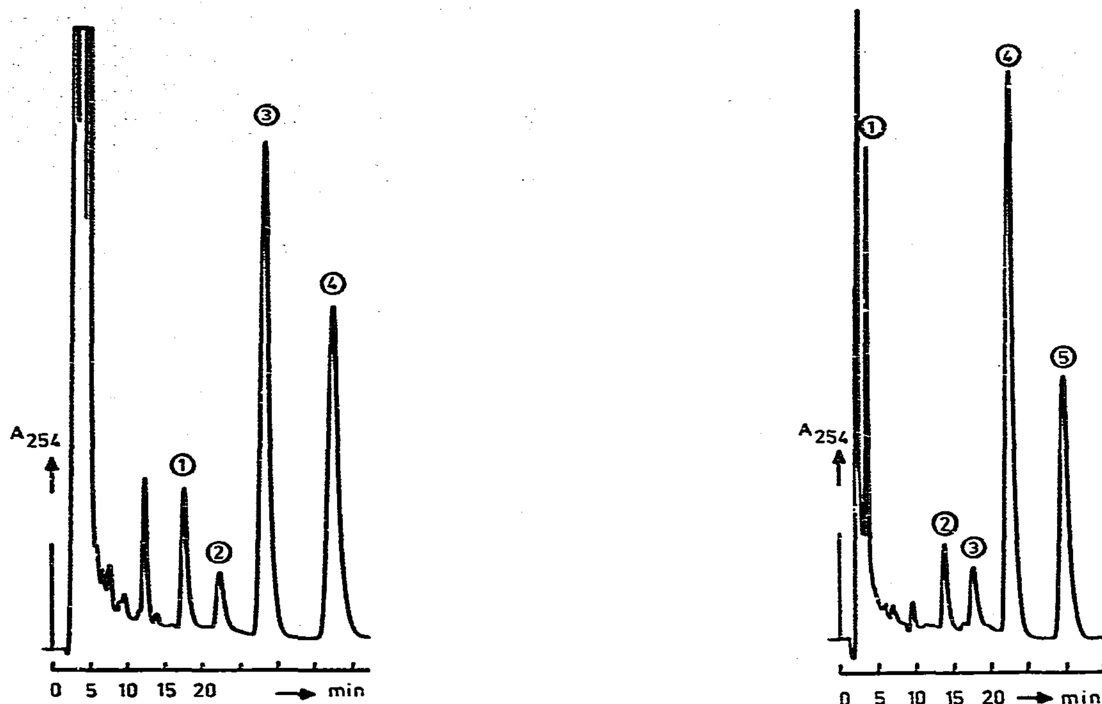

Fig. 5. Chromatogram of an extract from serum of patient B (10 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 2.9 µl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam; 4 = diazepam.

Fig. 6. Chromatogram of an extract from saliva of patient J (40 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 3.6 µl/sec. Peaks: 1 = caffeine; 2 = oxazepam; 3 = hydroxydiazepam; 4 = desmethyldiazepam; 5 = diazepam.

1.3% for 20 ng of diazepam injected to 0.2% for 1000 ng of oxazepam injected. The linearity of the calibration graphs, characterized by the correlation coefficients, was determined to be from 0.99990 (diazepam) to 0.99998 (oxazepam). The sensitivity of the system, defined as the slope of the graph of peak area versus amount injected expressed in integration units, ranged from 7.43 to 9.34 mV·sec/ng.

Fig. 4 shows the separation of a test mixture of diazepam and its metabolites, and it can be estimated that the limit of detection ranges from about 200 to 340 pg.

Application of the method to biological specimens

In order to test the method, serum, urine and saliva levels in volunteers and ambulant patients were determined. The results are given in Table IV.

No measurable amounts of desmethyldiazepam were seen after single doses of 5 or 10 mg, in contrast with chronic doses [14]. It appears from Table IV that after a single dose of 5 mg of diazepam, the serum levels of diazepam of the two volunteers after 2 h are about the same. However, subject R.M. showed remarkably high concentrations of desmethyldiazepam after both 2 and 9 h, which is more or less valid for the other metabolites. From a few ambulant pa-

SERUM, URINE AND SALIVA LEVELS OF DIAZEPAM AND ITS METABOLITES AFTER A SINGLE DOSE AND CHRONIC ADMINISTRATION

Ox = oxazepam, Hydr. = hydroxydiazepam, Des = desmethyldiazepam, D = diazepam.

Subject	Dose	Co-medication	Serum level (ng/ml)				Urine level (μg/ml)				Saliva level (ng/ml)			
			Ox.	Hydr.	Des	D	Ox.	Hydr.	Des	D	Ox.	Hydr.	Des	D
J.P.N.*	1 × 5 mg diazepam	—	det.	~1.2	0.8	63								
J.P.N.**	(single dose)		det.	~1.2	1.6	45								
R.M.*	1 × 5 mg diazepam	—	~6	~6	15	62								
R.M.**	(single dose)		~8	~4	16	34								
R.M.*	1 × 10 mg diazepam	—	~9	~8	29	243								
R.M.**	(single dose)		~8	~6	31	98								
J***	10 mg diazepam 4 dd§	Lorazepam (2 mg) Butobarbital (100 mg) Vibramycine Rhinathiol Antalby	132	130	1025	596	14.7	8.2	4.9	—	98	82	603	486
B	10 mg diazepam 1 dd	Paracetamol Lasix	109	53	392	419					272	173	136	71
C	5 mg diazepam 1 dd	Disipal Sinemet	48	20	107	99					—	25	9	21
L§§	30 mg oxazepam 1 dd						9.8	—	—	—				

*Sample taken 2 h after intake.
**Sample taken 9 h after intake.
***Total amount of urine: 910 ml in 24 h.
§dd = daily devided dose.
§§

tients serum, saliva and urine samples were analysed for diazepam and its metabolites. All of them received diazepam orally daily and used it chronically for at least 2 weeks. All of the patients also received other drugs. The urine was collected during 24 h, but there was no special time for taking the blood or saliva samples.

Table IV gives assays of serum levels for patients receiving various compounds as co-medication. Comparison of patient C, receiving 5 mg of diazepam daily, and the volunteers receiving the same dose (J.P.N. and R.M.) shows the great difference between the levels after single doses and chronic administration. The values in Table IV are in agreement with those given in the literature [14, 15].

Fig. 5 shows a chromatogram of an extract from serum of patient B. It can be seen that the co-medicants do not interfere with the compounds of interest.

Patient J is interesting because of the extensive co-medication involved. Many of the drugs used give acidic metabolites in blood and urine, some of which will not be extracted with the procedure described above, while others are separated chromatographically from the benzodiazepines. In none of these instances is the assay hampered by the co-medication.

The determination of saliva levels of benzodiazepines in comparison with urine and/or serum levels is interesting; however, only a few data are given in the literature [16, 17]. Fig. 6 shows a chromatogram of an extract from saliva of patient J (see Table IV).

It is notable that in the saliva from patient B the amounts of oxazepam and 3-hydroxydiazepam are higher than those of diazepam and nordiazepam,

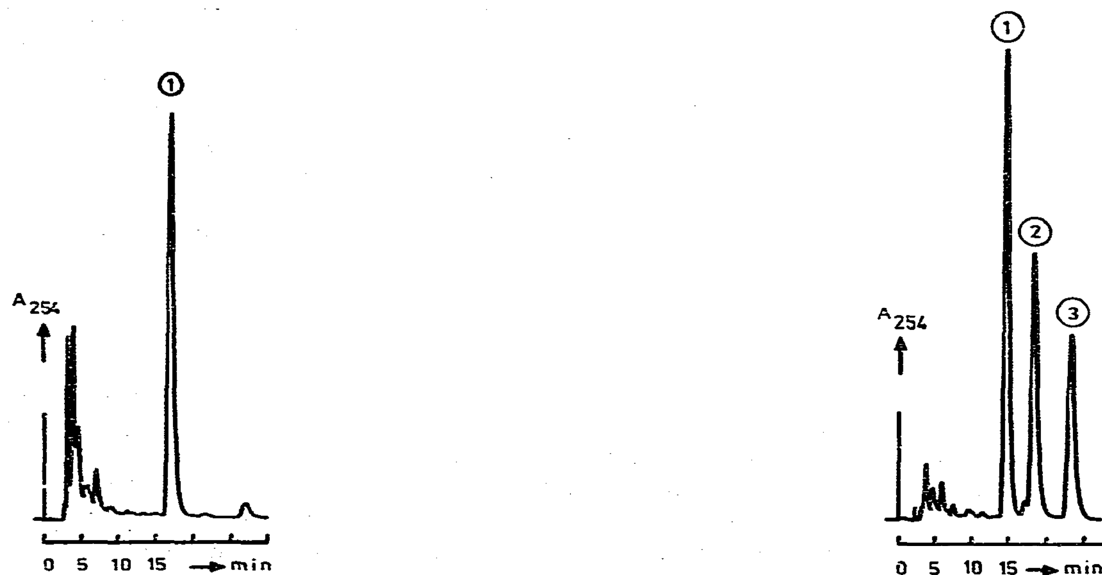

Fig. 7. Chromatogram of an extract from urine of patient L (30 mg of oxazepam daily). Conditions as in Fig. 4, except flow-rate = 2.9 µl/sec. Peaks: 1 = oxazepam.

Fig. 8. Chromatogram of an extract from urine of patient J (40 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 3.6 µl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam.

TABLE V

AMOUNTS OF FREE METABOLITES AND CONJUGATED METABOLITES IN URINE OF PATIENT J

Component	Concentration of free metabolite in urine (ng/ml)	Total amount in urine (ng/ml)	Percentage conjugated
Diazepam	10	—	—
Oxazepam	32	14,740	99.8
Desmethyldiazepam	76	4,990	98.5
3-Hydroxydiazepam	37	8,230	99.9

while in patient J the amounts of desmethyldiazepam and diazepam are much higher than those of oxazepam and 3-hydroxydiazepam. It can be concluded from Table IV that there are great individual differences in the serum to saliva ratio for diazepam and its metabolites, which indicates that further studies should be devoted to the utility of the determination of saliva levels in order to determine serum levels. An advantage is the greater availability of saliva than blood. Caffeine and theophylline substances from coffee usually present in saliva do not interfere with the benzodiazepines. A disadvantage could be the varying amounts of saliva delivered by the patients. The same occurs with single voidings of urine. Urine was collected from patients J and L during 24 h and then analysed (Table IV). Patient L received 30 mg of Seresta® (oxazepam) daily from which oxazepam glucuronide is the only urinary metabolite (see Fig. 7).

Fig. 8 shows a chromatogram of a urine extract from patient J. It can be seen that the results agree well with the literature [17]. Although most benzodiazepines are conjugated, small amounts of free benzodiazepines can be found in urine.

Table V gives the urine results for patient J, who was receiving 40 mg of diazepam daily.

CONCLUSIONS

It can be concluded that reversed-phase HPLC is a valuable technique for the assay of benzodiazepines and their metabolites in serum and saliva. As a consequence of the hydrolysis necessary with urine samples, the method is time consuming in this instance. The phase system shows a good selectivity towards all of the benzodiazepines examined, which means that by varying the pH practical problems can be solved. Future research will be devoted to the more rapid analysis of urine samples.

REFERENCES

1 M.A. Brodes, J.J. Bel Bruno, J.A.F. de Silva and H.R. Hackman, Anal. Chim. Acta, 74 (1975) 367.
2 M.A. Brooks and M.R. Hackman, Anal. Chem., 47 (1975) 2059.
3 M.A. Brooks and J.A.F. de Silva, Talanta, 22 (1975) 849.
4 M.M. Ellaithy, J. Volke and O. Manousek, Talanta, 24 (1977) 137.
5 J.A.F. de Silva and M. Strojny, J. Pharm. Sci., 60 (1971) 1303.
6 D. Maness and G.J. Yakatan, J. Pharm. Sci., 64 (1975) 652.
7 J.A.F. de Silva and C.V. Puglisi, Anal. Chem., 42 (1970) 1725.
8 I.A. Zingales, J. Chromatogr., 75 (1973) 55.
9 D.M. Hailey, J. Chromatogr., 98 (1974) 527.
10 P.M. Kabra, G.L. Stevens and L.J. Marton, J. Chromatogr., 150 (1978) 355.
11 R.R. Brodie, L.F. Chasseaud and T. Taylor, J. Chromatogr., 150 (1978) 361.
12 K. Harzer and R. Barchet, J. Chromatogr., 132 (1977) 83.
13 A. Bugge, J. Chromatogr., 128 (1976) 111.
14 J.A.F. de Silva, B.A. Koechlin and G. Bader, J. Pharm. Sci., 55 (1966) 692.
15 J.A. Zingales, J. Chromatogr., 75 (1973) 55.
16 K. Feller, G. Le Petit and U. Marx, Pharmazie, 31 (1976) 745.
17 P.J. Greenblatt and R.J. Shader, Benzodiazepines in Clinical Practice, Raven Press, New York, 1974, pp. 23–24.