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Overpressured layer chromatographic study of retention behaviour of various benzodiazepine derivatives on layers impregnated with tricaprylmethylammonium chloride*

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Abstract: The retention behaviour of various benzodiazepine derivatives was investigated on silica gel layers impregnated with tricaprylmethylammonium chloride (TCMA). The chromatograms were developed by means of overpressured layer chromatography (OPLC). As for the case of amino- and nitrosalicylic acids, pyrimidine derivatives, barbiturates, penicillins, cephalosporins and tetracyclines, the retention of benzodiazepine derivatives increased with increasing layer TCMA concentration with eluents containing methanol and water, but not TCMA. On increase of the methanol content of the eluent, a retention-decreasing effect was observed. On layers impregnated with TCMA, a linear relationship existed between the R_M values of the benzodiazepines and the methanol content of the eluent. A similar relationship held for silica gel layers impregnated with paraffin oil (traditional reversed-phase). There was no correlation between the results obtained on layers treated with TCMA or with paraffin oil. On TCMA-impregnated layers, the retention of compounds having different chemical structures showed no dependence on the pH of the eluent. There were two reasons for this. Firstly, as it was established, above a certain R_F value, the pH of the layer in the presence of TCMA was almost identical irrespective of the original pH of the buffer. Secondly, below this R_F value, the actual pH of the layer did not have a strong enough effect to cause appreciable differences between the retentions of the dissociated and undissociated species of the analytes. The conditions for optimum separation are given.

Keywords: *Benzodiazepines; tricaprylmethylammonium chloride; OPLC.*

Introduction

Benzodiazepine derivatives are of considerable importance as a consequence of their hypnotic, tranquillizing and anticonvulsant properties.

Numerous chromatographic techniques have been used for their identification and quantitation. In 1986, Chiarotti *et al.* [1] and later Sioufi and DuBois [2] critically surveyed and compared most of the chromatographic separation methods for benzodiazepines. Cserháti and his coworkers [3, 4] reported the retention behaviour of 18 benzodiazepine derivatives on traditional reversed-phase [3] and cyano, amino and diol HPTLC plates [4], with eluents suitable for both adsorption and reversed-phase separations, and they also investigated the relationship between R_M and the organic solvent content of the eluent for different benzodiazepines.

Recently studies have been made on the chromatographic behaviour of several acidic compounds, such as amino- and nitrosalicylic acids [5], penicillins and cephalosporins [6], barbiturates [7], the basic pyrimidine derivatives [8], and the amphoteric tetracyclines [9], using silica gel layers impregnated with tricaprylmethylammonium chloride (TCMA), with eluents containing methanol and water. The retention of such compounds is affected by the quantity of TCMA adsorbed on the silica gel. The retention increased with increasing TCMA concentration, and the organic solvent content of the eluent had a retention-decreasing effect, while the pH of the eluent and the presence of an inorganic salt (e.g. sodium chloride) had no effect on the retention.

The object of the present work was to study the retention characteristics of various benzodiazepine derivatives (Fig. 1) under similar circumstances and to seek a relationship between the R_M values and the methanol content of the eluent on silica gel layers impregnated with TCMA. The results are compared with those obtained on silica gel layers treated with paraffin oil (i.e. traditional reversed-phase).

For the development of the chromatograms overpressured layer chromatography (OPLC)

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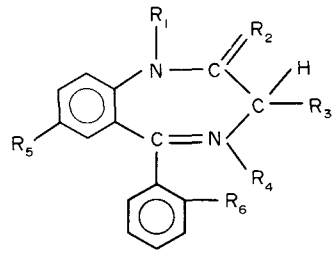
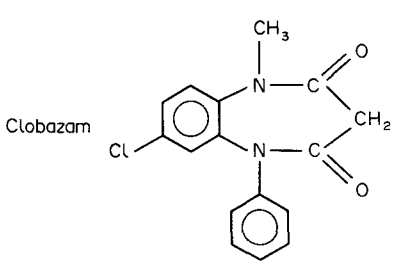
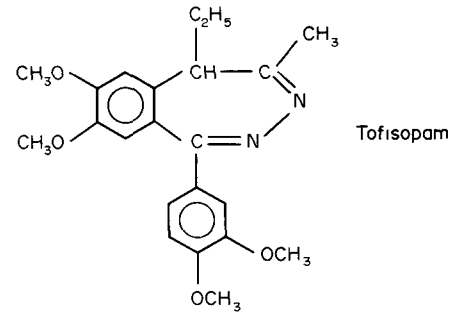
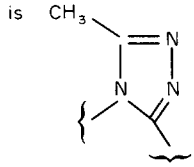


Compounds	R_1	R_2	R_3	R_4	R_5	R_6
Bromazepam	H	=O	H	H	Br	a
Uxepam	CH_3	=O	H	$CONH_2$	Cl	H
Oxazepam	H	=O	OH	H	H	H
Lorazepam	H	=O	H	H	Cl	Cl
Nitrazepam	H	=O	H	H	NO_2	H
Chlordiazepoxide	H	$NHCH_3$	H	O	Cl	H
Alprazolam	b	b	H	H	Cl	H
Clorazepat	H	$(OH)_2$	COOH	H	Cl	H
Diazepam	CH_3	=O	H	H	Cl	H
Medazepam	CH_3	H	H	H	Cl	H
Prazepam	c	=O	H	H	Cl	H





a 2 – Pyridinyl instead of phenyl group.
b part structure at R_1, R_2



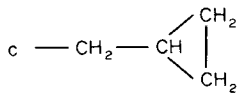
c 

Figure 1
Chemical structures of studied benzodiazepines.

developed by Tyihák and co-workers [10–12], was used. Using OPLC the chromatographic conditions are more reproducible than in a normal chamber, there being no vapour-space in OPLC. Also, one run takes only a few minutes and needs only a few millilitres of solvent.

Experimental

A Chrompres[25] OPLC instrument (Laboratory Instruments Co. Ltd, Budapest, Hungary) was used for the development of the chromatograms, at a membrane pressure of 20 bar and a flow rate of 1 ml min^{-1}.

The silica gel layers (Art. No. 5554) were obtained from E. Merck (Darmstadt, Germany). Three edges of the plates were impregnated with an Impres solution (Laboratory Instruments Co.). The layers were developed with methanolic TCMA solution in a normal chamber overnight.

For the traditional reversed-phase studies, the silica gel layers were impregnated with 5% paraffin oil in *n*-hexane. For reversed-phase experiments, RP-18 and RP-2 layers obtained from E. Merck (Art. Nos 13724 and 13726) were used. TCMA was purchased from Fluka (Basel, Switzerland). All other chemicals were of analytical grade.

The benzodiazepine derivatives were kindly provided by Dr S. Olajos (National Institute for Nervous and Mental Diseases, Budapest, Hungary). Two microlitre volumes of 1 mg ml^{-1} methanolic solutions were applied to the plates. The spots were visualized under a UV lamp at 254 nm.

For calculation of the resolution (R_S) and R_M values, the following expressions were used:

$$R_S = 2(Z_{x1} - Z_{x2})/(w_{b1} + w_{b2}), \quad (1)$$

where Z_x is the migration distance of the zone (spot) centre and w_b is the width of the zone (spot) at its base [13].

$$R_M = \log(1/R_F - 1). \quad (2)$$

Results and Discussion

Effects of TCMA concentration on retention

The effects of the TCMA concentration in the impregnating methanolic solution on the retention of the benzodiazepines were studied up to 0.2 M TCMA [Fig. 2(A) and (B)], with eluent containing methanol (30%) and water, but not TCMA. The R_F vs TCMA concentration curves of the compounds exhibit a similar tendency; the retention increases with increasing TCMA concentration. On bare silica gel, the derivatives generally move to the upper part of the layer; medazepam (R_F = 0.18), prazepam (R_F = 0.37) and bromazepam (R_F = 0.44) are exceptions. The presence of 0.005 M TCMA in the impregnating solution causes large increases in the retentions of lorazepam, clorazepate and nitrazepam. The retentions of the other derivatives are also larger at 0.005 M TCMA than on bare silica gel. Uxepam exhibits the largest, and medazepam the smallest R_F value at any TCMA concentration.

At higher than 0.05 M TCMA concentration, only small increases can be observed in the retentions with increasing TCMA concentration. At 0.2 M TCMA, the R_F values for all of the studied derivatives are smaller than 0.3.

This retention behaviour of benzodiazepines is very similar to that of amino- and nitrosalicylic acids [5], penicillins and cephalosporins [6], barbiturates [7], pyrimidine derivatives [8] and tetracyclines [9]. The similar retention behaviour indicates a similar retention mechanism, i.e. hydrophobic interactions play an important rôle in the separation of benzodiazepines on silica gel layers impregnated with TCMA.

Effects of eluent pH on retention

To study the effect of the pH of the eluent, silica gel layers impregnated with 0.005 M or 0.1 M TCMA were used with eluent containing methanol (30%) and aqueous Britton–Robinson buffer (0.2 M) at pH 2 or pH 10. As for the case of compounds having different chemical structures [5–9], the pH of the eluent has no effect on the retentions of the benzodiazepine derivatives.

For comparison, several experiments were performed on RP-2 and RP-18 layers by using eluents with the same composition. On reversed-phase layers at either pH 2 or pH 10, most of the derivatives showed no migration. On increase of the methanol content of the eluent from 30 to 50%, all of the benzodiazepines showed at least some migration. The retentions of medazepam, chlordiazepoxide and tofisopam were considerably lower at pH 10 than at pH 2. The retentions of nitrazepam, oxazepam, prazepam and uxepam also decreased, but to a lesser extent. For the other derivatives, the pH of the eluent had practically no effect on the retention on reversed-phase layers. The retentions of benzodiazepines are larger on RP-18 layers than on RP-2 layers.

To clarify the cause of this behaviour, the actual pH values of the silica gel layers impregnated with TCMA and those of the reversed-phase layers were measured. The solid substance was scraped off from four different sites (near to the starting zone, at R_F = 0.5, near to the frontal zone and from the undeveloped

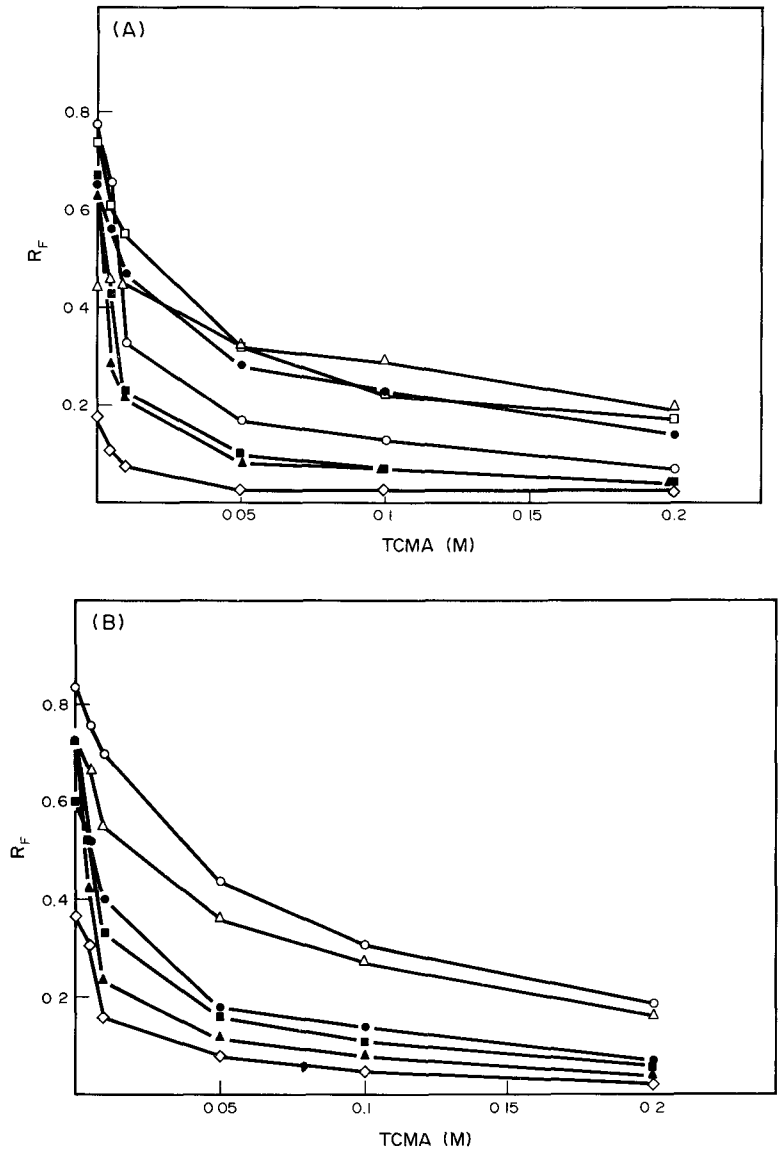


Figure 2
Effect of TCMA concentration in the impregnating solution on retention. (A) ●, alprazolam; △, bromazepam; ■, clorazepate; ◇, medazepam; ○, oxazepam; □, tofisopam. (B) ●, chlordiazepoxide; △, clobazam; ■, diazepam; ▲, nitrazepam; ◇, prazepam; ○, uxepam.

part) on the layer after development with eluents containing 50% of methanol and 0.2 M aqueous Britton–Robinson buffer at pH 2 or pH 10.

Seven millilitres of water were added to 0.1 g of layer material and the suspension was treated in an ultrasonic bath for 5 min. A pH gradient can be seen both on silica gel impregnated with TCMA and on RP-18 layers (Fig. 3). The actual pH of the layer at the starting zone is close to the pH value of the eluent, but the pH of the layer then decreases with increasing distance from the starting zone with eluent containing buffer of pH 10. For eluent containing buffer of pH 2, the pH of the layer increases with increasing distance from the starting zone. From $R_F = 0.5$, there are only slight differences between the pH values measured on layers impregnated with 0.005 or 0.1 M TCMA with eluents containing buffer of either pH 2 or pH 10. On RP-18 layers, the difference is about 2.2 pH units in the same position. This explains why there was a pH-dependence of the retentions on reversed-phase layers and not on silica gel layers impregnated with TCMA. On reversed-phase layers, the R_F values of the benzodiazepines are low (the maximum is 0.33) with eluent

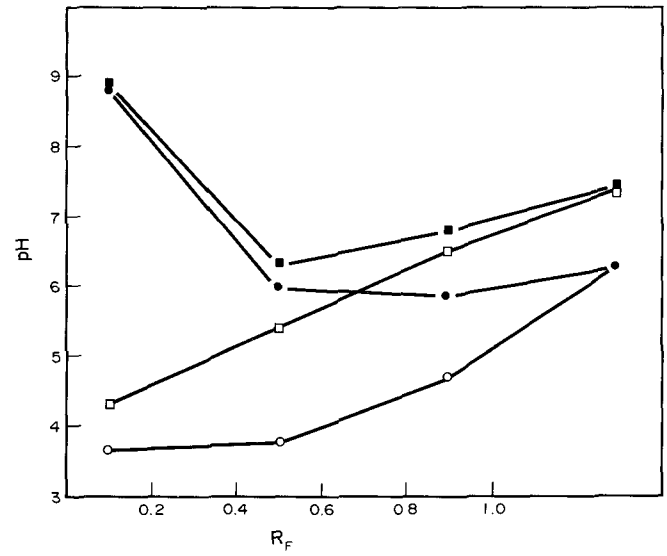


Figure 3
Actual pH values on silica gel layers impregnated with 0.1 M TCMA and RP-18 layers, with eluents containing a buffer of pH 2 or pH 10. □, Silica gel at pH 2; ■, silica gel at pH 10; ○, RP-18 layer at pH 2; ●, RP-18 layer at pH 10.

containing 50% of methanol at pH 2. On silica gel layers impregnated with 0.1 M TCMA, the R_F values are in the range 0.32–0.67 with the same eluent. TCMA adsorbed on the silica gel layer may contribute to the effect of the buffer used in the eluent. The pH of the layer is the same above a certain R_F value. Below this the actual pH of the layer does not have sufficient effect to cause appreciable differences between the retentions of the dissociated and undissociated species of the analytes.

Effects of methanol content of eluent; R_M *versus methanol concentration curves*

Again, as the compounds studied earlier [5–9], for the retentions of the benzodiazepines decrease with increasing methanol content of the eluent.

On the basis of the work of Cserháti *et al.* [3, 4], who studied the relationship between the R_M values and the organic solvent content of the eluent for benzodiazepine derivatives on different types of plates, it was decided to investigate whether such a correlation holds on silica gel impregnated with 0.005 or 0.124 M (5%) TCMA solutions. For calculations the following equation was used:

$$R_M = a + bC, \qquad (3)$$

where C is the methanol concentration (v/v %) of the eluent.

The two TCMA concentrations used represent a partial and an almost full coverage of the silica surface. On layers impregnated with 0.005 M TCMA, the methanol concentration of the eluent varied from 10 to 50%, while on layers impregnated with 0.124 M TCMA the effect of the methanol content of the eluent was studied in the range 30–60%. With a larger concentration of TCMA for impregnation, the retentions of the benzodiazepines are larger at a given methanol concentration. On increase of the methanol content of the eluent, the retention decreases, and consequently the R_F value increases. The results obtained were similar to those of Cserháti [4]. The R_M vs methanol concentration relationships are linear (Table 1) on layers impregnated with either 0.005 or 0.124 M TCMA. The R_M values decrease with increasing methanol content of the eluent. The experimental data fit well, especially on a layer impregnated with 0.124 M TCMA. This correlation indicates that the benzodiazepine derivatives do not exhibit any anomalous retention behaviour on silica gel layers covered partially or fully with TCMA.

The results obtained in the presence of TCMA on the layers gave similar R_M vs methanol concentration curves to those for silica gel layers impregnated with 5% paraffin oil in *n*-hexane (Table 1).

On traditional reversed-phase layers, the relationships between the R_M values and the

Table 1
Equations and r values of linear correlations between the R_M for benzodiazepines and methanol content of eluent on silica gel layers impregnated with TCMA or paraffin oil

Compound	0.005 M TCMA		0.124 M TCMA		5% Paraffin oil	
	$R_M = a + bC$	r	$R_M = a + bC$	r	$R_M = a + bC$	r
Bromazepam	0.852–0.0272C	0.9981	2.866–0.0511C	0.9962	5.601–0.1050C	0.9944
Uxepam	0.466–0.0306C	0.9915	2.949–0.0593C	0.9965	5.403–0.1042C	0.9913
Oxazepam	1.172–0.0360C	0.9900	5.503–0.0949C	0.9965	5.395–0.1001C	0.9991
Nitrazepam	1.418–0.0380C	0.9809	5.754–0.0937C	0.9999	5.248–0.1002C	0.9983
Chlordiazepoxide	1.062–0.0318C	0.9873	5.242–0.0915C	0.9967	7.490–0.1260C	0.9931
Alprazolam	0.668–0.0244C	0.9939	3.729–0.0694C	0.9944	7.335–0.1213C	0.9912
Chlorazepate	1.625–0.0439C	0.9768	6.094–0.1000C	0.9976	6.379–0.1082C	0.9994
Diazepam	1.170–0.0358C	0.9779	5.372–0.0936C	0.9997	7.451–0.1219C	0.9978
Medazepam	2.265–0.0451C	0.9887	6.751–0.0978C	0.9980	very low R_F values!	
Prazepam	1.759–0.0419C	0.9820	6.630–0.1069C	0.9987	8.134–0.1235	0.9997
Clobazam	0.710–0.0310C	0.9930	3.380–0.0639C	0.9841	5.964–0.1105C	0.9969
Tofisopam	0.876–0.0324C	0.9933	3.846–0.0762C	0.9927	7.638–0.1349C	0.9836

methanol content of the eluent are also linear and have a negative slope. However, there is no linear correlation between the data obtained on TCMA-impregnated layers and on paraffin oil-impregnated layers. A possible cause is that there are differences in the sequence of the retention of the derivatives on layers impregnated with TCMA compared with layers impregnated with paraffin oil (Table 2). These differences are especially considerable for nitrazepam, tofisopam and alprazolam. On the other hand, for uxepam, prazepam and medazepam the sequence of the R_F values is the same on the two layers. The fact that the methanol content of the eluent can desorb a certain quantity of TCMA from the layer [6–9], while paraffin oil is practically insoluble in methanol, may be pertinent to this phenomenon. A study of the adsorption of paraffin oil on silica gel layers is currently in progress.

Table 2
R_F values of benzodiazepines on silica gel layers impregnated with 5% TCMA or with 5% paraffin oil using an eluent containing 50% of methanol

Compound	R_F on silica gel layers impregnated with 5% TCMA	5% Paraffin oil
Medazepam	0.14	0.05
Prazepam	0.23	0.12
Nitrazepam	0.25	0.45
Clorazepate	0.26	0.28
Diazepam	0.33	0.21
Oxazepam	0.33	0.41
Chlordiazepoxide	0.34	0.24
Bromazepam	0.43	0.42
Alprazolam	0.45	0.22
Clobazam	0.49	0.39
Tofisopam	0.50	0.30
Uxepam	0.52	0.48

Optimum separations on TCMA-impregnated layers

The separation on silica gel layers impregnated with TCMA can be affected by the TCMA quantity adsorbed on the silica surface. It is controlled directly by the TCMA concentration in the impregnating solution and indirectly by the methanol content of the eluent, through a desorbing effect. All the possible R_S (resolution) values (78 for 13 components) were calculated for silica gel layers impregnated with 0.005 M TCMA, using an eluent containing 30% of methanol. In 65 cases, the R_S values are larger than 1 (Table 3). In eight of the remaining 13 cases, $R_S > 1$, if the layers are impregnated with 0.01 M TCMA. In an additional three cases, separation can be achieved on a non-impregnated silica gel layer. There is no resolution between clobazam and tofisopam or between clorazepate and nitrazepam in any of the chromatographic systems mentioned above.

For comparison, the R_S values were calculated on layers impregnated with 5% paraffin oil in *n*-hexane, for which the best separation can be achieved with an eluent containing 60% of methanol (45 cases).

These results permit the conclusion that the presence of TCMA on the silica gel layer improved the resolution of the benzodiazepine derivatives.

Table 3
R_S values on silica gel layers impregnated with 0.005 M TCMA with an eluent containing 30% of methanol

Compound	R_S	Compound	R_S	Compound	R_S	Compound	R_S	Compound	R_S	Compound	R_S
ALP/BRO	1.4	BRO/CLO	3.0	DIA/CLD	<1	CLO/PRA	4.4	LOR/NIT	1.6	MED/PRA	2.9
ALP/DIA	<1	BRO/CLR	<1	DIA/MED	6.7	CLO/TOF	<1	LOR/OXA	4.9	MED/TOF	7.8
ALP/CLO	1.5	BRO/LOR	2.5	DIA/NIT	1.2	CLO/UXE	1.4	LOR/PRA	<1	MED/UXE	10.7
ALP/CLR	1.7	BRO/CLD	<1	DIA/OXA	1.6	CLR/LOR	1.9	LOR/TOF	4.4	NIT/OXA	2.5
ALP/LOR	3.9	BRO/MED	5.7	DIA/PRA	2.5	CLR/CLD	1.2	LOR/UXE	6.6	NIT/PRA	1.1
ALP/CLD	<1	BRO/NIT	<1	DIA/TOF	1.1	CLR/MED	4.8	CLD/MED	6.8	NIT/TOF	2.1
ALP/MED	7.5	BRO/OXA	2.6	DIA/UXE	3.1	CLR/NIT	<1	CLD/NIT	1.1	NIT/UXE	3.8
ALP/NIT	1.7	BRO/PRA	1.7	CLO/CLR	3.2	CLR/OXA	2.7	CLD/OXA	1.8	OXA/PRA	3.9
ALP/OXA	1.2	BRO/TOF	2.1	CLO/LOR	5.7	CLR/PRA	1.3	CLD/PRA	2.5	OXA/TOF	<1
ALP/PRA	3.0	BRO/UXE	4.2	CLO/CLD	2.1	CLR/TOF	2.3	CLD/TOF	1.2	OXA/UXE	1.3
ALP/TOF	<1	DIA/CLO	1.9	CLO/MED	9.8	CLR/UXE	4.2	CLD/UXE	3.3	PRA/TOF	3.5
ALP/UXE	2.7	DIA/CLR	1.3	CLO/NIT	2.9	LOR/CLD	3.4	MED/NIT	4.0	PRA/UXE	5.4
BRO/DIA	<1	DIA/LOR	3.4	CLO/OXA	<1	LOR/MED	2.8	MED/OXA	8.4	TOF/UXE	1.9

ALP, alprazolam; BRO, bromazepam; DIA, diazepam; CLO, clobazam; CLR, clorazepate; LOR, lorazepam; CLD, chlordiazepoxide; MED, medazepam; NIT, nitrazepam; OXA, oxazepam; PRA, prazepam; TOF, tofisopam; UXE, uxepam.

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