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Short communication

Rapid and sensitive detection of benzodiazepines and zopiclone in serum using high-performance thin-layer chromatography

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Abstract

We developed a rapid and sensitive method of identifying benzodiazepines and zopiclone in human serum using high-performance thin-layer chromatography (HPTLC). These drugs were developed and separated on plates within 8–11 min and detected by means of UV radiation and colour. Each drug was accurately identified by means of the values of $R_F \times 100$ and the spot colour in three systems. The detection limit of the benzodiazepines in serum was 0.1–0.4 μg/ml, except for cloxazolam and haloxazolam. The sensitivity was increased about ten-fold over the conventional method. These results suggested that the HPTLC system is useful for the initial detection and identification of these drugs in emergencies.

1. Introduction

Among various chromatographic methods, thin-layer chromatography (TLC) is comparatively simple, rapid and convenient for identifying many chemicals. It has been applied to the identification of drugs which induce poisoning or abuse. Benzodiazepines and zopiclone often induce coma at large doses, and may mask the actions of other drugs taken concomitantly. In emergencies, it is most important for clinicians to identify specific drugs rapidly, to make decisions as to treatment. The conventional method for identification in emergencies has been the Toxi-Lab system, including TLC. However, the detection limits in serum are low enough to identify several benzodiazepines [1]. In this study, we developed a useful method for identifying benzodiazepines in human serum in emergencies, using rapid and sensitive high-performance thin-layer chromatography (HPTLC).

2. Experimental

2.1. Apparatus and reagents

The HPTLC system consisted of a Camag Horizontal Development Chamber (Muttenz, Switzerland), pre-coated silica gel 60 F254 HPTLC plates (Merck Art. 5628, 10 × 10 cm) (Merck, Darmstadt, Germany), a 1-μl Hamilton microsyringe (Hamilton, Reno, NV, USA) and a fluorescence inspection lamp, Fi-5S (Topcon,

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Tokyo, Japan). The high-performance liquid chromatography (HPLC) system consisted of an LC-10A pump, an LC-10AD detector with a Chromatopac C-R7A data processor (Shimadzu, Kyoto, Japan), and a LichroCART RP-18 column (4.6 × 150 mm, 5 μm; Cica-MERCK, Tokyo). Drug-free lyophilized serum was purchased from Bio-Rad (Anaheim, CA, USA) to prepare control and standard drug sera. The drugs were supplied by the following companies: bromazepam, flunitrazepam and flurazepam from Hoffman-La Roche (Basel, Switzerland); chlordiazepoxide, diazepam and estazolam from Takeda (Osaka, Japan); brotizolam from Boehringer (Ingelheim, Germany); cloxazolam and haloxazolam from Sankyo (Tokyo); alprazolam and triazolam from Upjohn (Kalamazoo, MI, USA); lormetazepam from Schering (Berlin, Germany); clotiazepam and etizolam from Yoshitomi (Osaka); medazepam and nitrazepam from Shionogi (Osaka); zopiclone from Rhône-Poulenc Rorer (Antony Cedex, France). All other chemicals were purchased from Wako (Osaka).

2.2. Standard drug serum preparation

Standard drug solutions were prepared at a concentration of 1 μg/ml in methanol. One millilitre of the standard was transferred into a test tube and evaporated at 25°C in vacuo. The residue was reconstituted in 1 ml of drug-free serum to prepare the standard drug serum, which was further diluted with the drug-free serum if necessary.

2.3. Extraction

An aliquot of 1 ml of 0.5 *M* phosphate buffer (pH 9.0) was added to the standard serum (1 ml) to adjust the pH, and shaken with 3 ml of *n*-hexane–ethyl acetate (75:25, v/v) for 10 min. After centrifugation at 3500 rpm (1800 *g*) for 5 min, the organic phase was transferred into a glass tube and evaporated to dryness at 25°C in vacuo. The residue was reconstituted in 10 μl of methanol and a 1 μl aliquot was applied 1 cm from the side of the HPTLC plate. The drug-free serum was used as a blank.

2.4. Development

We used the following developing solvent systems as reported [2]: system 1, cyclohexane–toluene–diethylamine (75:15:10, v/v/v); system 2, chloroform–methanol (90:10, v/v); and system 3, chloroform–acetone (80:20, v/v). The plates were developed in a sandwich configuration, in Camag Development Chambers, from both sides towards the middle of the plate. The plate was then dried at room temperature.

2.5. Detection

The separated spots on the plate were first placed under UV radiation at wavelengths of 254 and 366 nm. Next, the plate was dipped into concentrated sulphuric acid and the spots were observed under UV radiation at 366 nm. Finally, the plate was treated with modified Dragendorff's reagent to visualize the spots and their R_F values were measured.

2.6. Recovery test

The recovery of the extraction procedure was measured using HPLC. The mobile phase was a mixture of methanol–water–phosphate buffer (0.1 *M*, pH 5.7) (60:20:20 or 65:15:20, v/v/v). The flow-rate was 0.75 ml/min. The recovery ratio was calculated from the peak heights obtained from the intrinsic standard and the extraction sample.

3. Results and discussion

Table 1 shows the $R_F \times 100$ values of sixteen benzodiazepines and zopiclone in the HPTLC system. In system 3, bromazepam was observed as a tailing spot and haloxazolam appeared as a streak. The HPTLC system seemed to be reproducible, as the standard deviation (S.D.) of $R_F \times 100$ values was small. These $R_F \times 100$ values were similar to those determined by TLC

Table 1
$R_F \times 100$ values and S.D. of benzodiazepines and zopiclone in three development systems (each value of $R_F \times 100$ is the mean of ten determinations)

Compound	System 1		System 2		System 3	
	$R_F \times 100$	S.D.	$R_F \times 100$	S.D.	$R_F \times 100$	S.D.
Alprazolam	1	0.40	46	2.64	6	0.59
Bromazepam	3	0.44	42	1.71	6[a]	0.74
Brotizolam	6	0.42	52	3.49	13	0.13
Chlordiazepoxide	1	0.41	49	2.94	11	0.84
Clotiazepam	38	1.55	66	3.49	60	1.13
Cloxazolam	2	0.71	47	3.06	41	2.74
Diazepam	29	1.13	65	2.76	59	0.98
Estazolam	1	0.07	42	1.37	9	0.74
Etizolam	4	0.18	45	0.56	13	0.76
Flunitrazepam	14	0.97	66	1.55	56	0.92
Flurazepam	34	1.07	42	2.46	6	0.75
Haloxazolam	2	0.14	44	2.35	13[b]	0.81
Lormetazepam	8	0.50	59	3.03	49	0.56
Medazepam	50	0.98	69	3.26	62	1.35
Nitrazepam	0	0.75	49	3.43	35	0.86
Triazolam	1	0.38	43	2.46	6	0.65
Zopiclone	21	0.91	71	2.54	60	0.98

[a] Tailing spot.
[b] Streaked spot.

[2,3], although the values for brotizolam, etizolam or zopiclone were not described. One exception was that we found the $R_F \times 100$ value for cloxazolam in development system 2 to be 47 compared with 3 in a previous study [2]. The latter value appears to be a mistake, because we confirmed repeatedly that the values of all substances in system 2 were larger than those in system 3.

Discrimination power is one of indices with which to evaluate the separating capability of chromatographic systems, and it is defined as the probability that two randomly selected substances can be separated in the system. The values of discrimination power calculated according to a previous report [4], where $3 \times$ S.D. of the measured $R_F \times 100$ values was used as the error factor, were 0.83, 0.57 and 0.88 in systems 1, 2 and 3, respectively. Considering this together with the $R_F \times 100$ values in these systems may be useful for detecting and identifying a specific drug among the benzodiazepines and zopiclone.

The colours of these drugs in the three development systems are shown in Table 2. The intrinsic responses to UV radiation, those after exposure to sulphuric acid, and those to modified Dragendorff's reagent were different. The sensitivity of the response to UV radiation is increased by treating benzodiazepines with concentrated acids, because of the formation of highly fluorescent derivatives [5]. We found that dipping the plate into concentrated sulphuric acid increases the detection sensitivity of benzodiazepines, especially chlordiazepoxide and etizolam. These results suggested that a combination of colours and the $R_F \times 100$ values is useful for detecting and identifying benzodiazepines and zopiclone in serum.

The detection limits of HPTLC for benzodiazepines and zopiclone are shown in Table 3. The detection limits of UV radiation for the standards in methanol were 10–50 ng per spot. The colouration conferred by the modified Dragendorff's reagent was slightly less sensitive in some cases. The detection limits of the drugs in serum were

Table 2
Colours of benzodiazepines and zopiclone developed by HPTLC systems

Compound	Colour		
	Stage I	Stage II	Stage III
Alprazolam	Violet/neg.[a]	Pale blue-green	Brown
Bromazepam	Violet/neg.	Pale brown	Pale brown
Brotizolam	Violet/neg.	Yellow	Pale brown
Chlordiazepoxide	Violet/Orange	Bright yellow	Pale brown or neg.
Clotiazepam	Violet/Yellow	Yellow	Pale brown
Cloxazolam	Violet/Yellow	Pale Brown	Pale brown or neg.
Diazepam	Violet/neg.	Yellow	Pale brown
Estazolam	Violet/neg.	Pale blue-green	Brown
Etizolam	Violet/Yellow	Bright yellow	Brown
Flunitrazepam	Violet/neg.	Pale blue-green	Pale brown
Flurazepam	Violet/neg.	Yellow	Brown
Haloxazolam	Violet/neg.	Faint (yellowish) or neg.	Pale brown or neg.
Lormetazepam	Violet/neg.	Yellow	Pale brown
Medazepam	Violet/neg.	Pale green	Pale brown
Nitrazepam	Violet/neg.	Pale blue	Pale brown
Triazolam	Violet/neg.	Pale blue-green	Pale brown
Zopiclone	neg./Orange	neg.	Pale brown or neg.

Detection methods: Stage I, UV radiation (254/366 nm); Stage II, UV radiation (366 nm) after dipping in concentrated sulphuric acid; Stage III, reaction with modified Dragendorff's reagent.
[a] neg. = fluorescence or colour not observed.

Table 3
Detection limits of benzodiazepines and zopiclone

Compound	In methanolic solution (ng/charged)		In serum (ng/ml) Stage I
	Stage I	Stage III	
Alprazolam	20	20	200
Bromazepam	40	40	400
Brotizolam	20	30	100
Chlordiazepoxide	25	50	100
Clotiazepam	30	30	100
Cloxazolam	20	50	800
Diazepam	15	30	200
Estazolam	20	50	200
Etizolam	15	30	100
Flunitrazepam	20	40	400
Flurazepam	10	25	200
Haloxazolam	30	60	1000
Lormetazepam	20	30	400
Medazepam	10	40	200
Nitrazepam	20	50	100
Triazolam	15	30	200
Zopiclone	10	20	100

0.1–1.0 μg/ml. This sensitivity may be sufficient to detect these drugs in patient serum after poisoning or abuse.

Here, we used an organic mixture (*n*-hexane–ethyl acetate = 75:25, v/v) as the extraction solvent as described [6]. The recovery ratios of the drugs from serum during the extraction procedure are shown in Table 4. The values for cloxazolam and haloxazolam could not be calculated, because the peaks were remarkably unstable in the chromatograms. This may be due to the continuous hydrolytic cleavage of benzodiazepinooxazole rings of cloxazolam and haloxazolam in the aqueous mobile phase [7]. The ratios of several drugs were 50–60%, but the coefficients of variation were as low as 6%. This organic mixture may be reasonably sufficient for extracting the drugs from serum.

Table 4
Recovery ratios of benzodiazepines and zopiclone with *n*-hexane–ethyl acetate (75:25, v/v) from serum

Compound	Recovery (%)	C.V. (%)
Alprazolam	54.65	2.67
Bromazepam	63.22	1.30
Brotizolam	76.82	3.92
Chlordiazepoxide	81.66	3.83
Clotiazepam	92.28	2.71
Cloxazolam	N.D.	–
Diazepam	95.57	1.43
Estazolam	57.12	1.56
Etizolam	70.00	3.59
Flunitrazepam	83.06	4.05
Flurazepam	76.08	5.35
Haloxazolam	N.D.	–
Lormetazepam	86.89	2.95
Medazepam	99.19	4.29
Nitrazepam	77.88	3.52
Triazolam	55.78	5.00
Zopiclone	98.43	1.49

Compared with a peak height of the intact standard ($n = 5$).
N.D. = not detectable.

The HPTLC system yielded a better separation and more rapid development than conventional TLC. We found that HPTLC is more sensitive and discriminatory than the conventional means of detecting benzodiazepines. This method would permit an easy detection and identification of benzodiazepine drugs and zopiclone in emergencies, and may help physicians decide how to treat patients after poisoning or abuse.

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