ADLİ TIP DERGİSİ
Journal of Forensic Medicine
Adli Tıp Dergisi 1990; 6(1-2): 3-8

TLC-Screening of 1,4-Benzodiazepines

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1,4-BENZODİAZEPİNLERİN İNCE TABAKA KROMATOGRAFİSİ İLE BELİRTİMİ

Özet

İnce tabaka kromatografisi (TLC) benzodiazepinlerin ve bunların metabolitlerinin taranması amacıyla yaygın olarak kullanılmaktadır. Yöntemin prensibi, hidrolizle elde edilen aminobenzofenon türevlerinin çekitlendikten sonra TLC ile ayırılmasına ve fotolitik olarak dezalkillenmesine dayanır. Diazolandırılmış ürünler azo boyalarıyla kenetlenir (örneğin, Bratton-Marshall ayıracı). Yöntem, çok sayıda benzodiazepine uygulanmış (1-5) ve özgüllüğü kanıtlanmıştır (6).

Summary

Thin-layer chromatography (TLC) is the preferred method for screening benzodiazepines and their metabolites. The procedure involves hydrolysis to yield aminobenzophenone derivatives, which are then extracted, separated by TLC and photolytically dealkylated. The products are diazotized and coupled with azo-dye (e.g. the Bratton-Marshall reagent). The method has already been applied to numerous benzodiazepines (1-5) and its specificity established (6).

Key words : *Benzodiazepines - TLC - Hydrolysis - Photolytic dealkylation - Diazotization*

EXPERIMENTAL

Reference Substances

ABFB	2-amino-5-bromo-2'-fluorobenzophenone (e.g. from haloxazolam)
ABP	(2-amino-5-bromophenyl) (2-pyridyl) methanone (e.g. from bromazepam)
ACB	2-amino-5-chlorobenzophenone (e.g. from oxazepam)
ACFB	2-amino-5-chloro-2'-fluorobenzophenone (e.g. from desalkylflurazepam)
ADB	2-amino-2'-5-dichlorobenzophenone (e.g. from lorazepam)
ANB	2-amino-5-nitrobenzophenone (e.g. from nitrazepam)
ANCB	2-amino-2'-chloro-5-nitrobenzophenone (e.g. from clonazepam)
ANFB	2-amino-2'-fluoro-5-nitrobenzophenone (e.g. from 1-desmethyl-flunitrazepam)
CCB	5-chloro-2- [(cyclopropylmethyl) amino] benzophenone (e.g. from prazepam)
CFMB	5-chloro-2'-fluoro-2-(methylamino) benzophenone (e.g. from fludiazepam)
CFTB	5-chloro-2'-fluoro-2- (2,2,2-trifluoroethylamino) benzophenone (e.g. from quazepam)
CPB	5-chloro-2- (2-propinyl) benzophenone (e.g. from pinazepam)
MACB	5-chloro-2- (methylamino) benzophenone (e.g. from diazepam)
MDB	2', 5-dichloro-2- (methylamino) benzophenone (e.g. from lormetazepam)
MNB	2- (methylamino) -5-nitrobenzophenone (e.g. from nimetazepam)
MNFB	2'-fluoro-2- (methylamino) -5-nitrobenzophenone (e.g. from flunitrazepam)
TCB	5-chloro-2- (2,2,2-trifluoroethylamino) benzophenone (e.g. from halazepam)

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Spray Solution (Bratton-Marshall Reagent)

Dissolve 1 g of N-(1-naphthyl)ethylenediamine in a mixture of 50 ml of dimethylformamide and 50 ml of 4 M hydrochloric acid, with warming if necessary. Filter the cooled solution if it is not clear. A slight violet colour does not affect its use. If kept in the refrigerator the solution is stable for about a year.

Standard Solution for TLC

Dissolve 1 mg each of the reference substances in 2 ml of methanol. Note that for screening not all the reference substances are absolutely necessary, but ANB, ACB, MACB and TCB should all be used for comparison purposes and for calculation of the corrected R_f value. If stored in glass bottles in the refrigerator (4°C) and protected from light, the solution is stable for several months.

To avoid interference in the TLC, no other substances should be present that give a colour with the Bratton-Marshall reagent.

Hydrolysis

Place 100 ml of the urine sample in a 500 ml erlenmeyer flask and add 50 ml of concentrated hydrochloric acid. Heat the mixture for 30 min under a reflux condenser, in a boiling water-bath, and if necesary rinse the condensate from the condenser into the flask with a little concentrated hydrochloric acid.

Neutralization and Extraction

After the hydrolysis, cool the solution to room temperature, and then, with further cooling, adjust the pH to between 8 and 9 (universal indicator paper) by addition of 8 M sodium hydroxide (about 5 ml or so will be needed). Wear safety goggles during this operation, which should be conducted under an efficient fume-hood on account of the very unpleasant smell. Extract the aminobenzophenone derivatives with about 200 ml of diethyl ether. Note that the acid hydrolysis of bromazepam and its metabolites yields not benzophenone derivatives, but only benzoylpyridine compounds, but as these behave like primary aromatic amines, they can be detected with Bratton-Marshall reagent. To increase the yield, the extraction can be repeated with 100 ml of diethyl ether, at pH 11. Reduce the combined extracts to a volume of about 3 ml in a rotary evaporator, and transfer this concentrate to a glass-stoppered centrifuge tube and carefully evaporate it to dryness (at about 30-40°C; it is not necessary to use reduced pressure). Cool the residue to 4°C and reserve it for analysis; for this dissolve it in 0.1 ml of methanol.

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Thin-Layer Chromatography

Use 20x20 cm Kieselgel 60 F_{254} TLC plates, layer thickness 0.25 mm. Apply the sample and standard spots 1.5 cm from the lower edge of the plate, with 2-µl capillaries. For each sample use three capillary-loads overlapped to give an approximately straight line of sample. To avoid any cross-contamination apply the test solutions before the standards. Run the chromatogram until the solvent front has travelled 10 cm. Use the ascending method, without chamber saturation. No special activation of the plates is needed, and would not improve the results anyway. Use about 100 ml of toluene as the mobile phase.

Photolytic Dealkylation

After the development of the chromatogram (which takes 30-40 min), leave the plate to drip in the development tank for a short time, then dry it in a cold air-stream under the fume-hood. Expose the dried plate to a suitable ultraviolet source (e.g. a sun-lamp) at a distance of 30-40 cm for about 20 min. For rapid analysis a 6 min exposure is sufficient. Immediately cool the plate to room-temperature, or the yield in the diazotization step will be impaired.

Note that the dealkylation step is only necessary when testing for diazepam, camazepam, temazepam, ketazolam, prazepam, flurazepam, flunitrazepam, lormetazepam, fludiazepam, nimetazepam, pinazepam, quazepam and halazepam (3).

Diazotization

Place the cool dry plate in an empty chromatographic tank, on the bottom of which is a small beaker (20-50 ml) containing 10 ml of 20 % sodium nitrite solution. Pipette 5 ml of 25 % v/v hydrochloric acid into the beaker as fast as possible, to liberate nitrogen oxides, and seal the tank with its lid. Leave the plate in the tank for 3-5 min, which is sufficient time for diazotization of primary aromatic amine groups. Remove the lid, and when most of the nitrous gases have dispersed take out the plate and leave it under the fume-hood for 20-30 min in a stream of cold air (e.g. from a fan heater set at "cold"). For rapid work it is sufficient to air the plate for only 5 min to remove the nitrous gases and then to spray it gently with a 1 % aqueous solution of urea. Finally spray the plate thinly and uniformly (meander pattern) with Bratton-Marshall reagent at 4°C to couple the diazonium salts. The result: violet and redviolet colours.

RESULTS

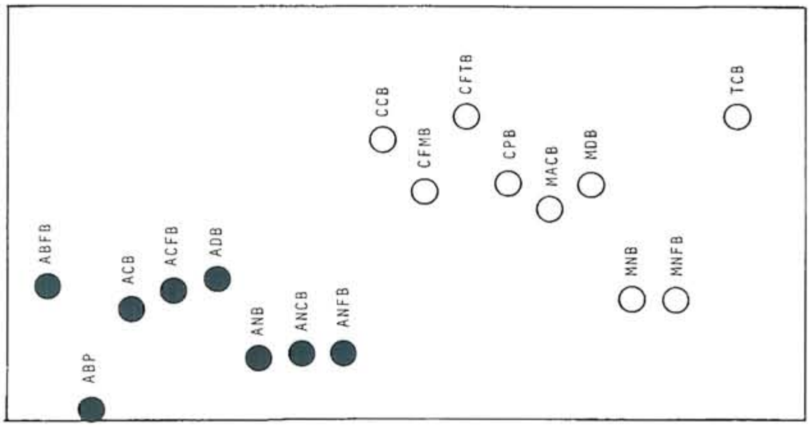

Figure 1. hR_f^c-values of 17 hydrolysis derivatives of commonly used 1,4-benzodiazepines (Stationary phase: Silicagel; Solvent-system: Toluene) (● = detectable without photolytical desalkylation ; ○ = detectable with photolytical desalkylation prior to diazotization).

Corrected R_f-Values of Hydrolysis Products of Benzodiazepines

The corrected hR_f-values of commonly used benzodiazepines using the 10 standardized TLC-systems are listed in Table I. However some special benzophenones (e.g. ACB from oxazepam, ACFB from desalkylflurazepam and ADB from lorazepam) can hardly be distinguished when using the systems 1 to 10. So we developed an additional system (=system 11) using toluene as solvent, silica as adsorbent and the following reference compounds:

ANB hR_f^c = 15
ACB = 27
MACB = 52
CCB = 68

Table I. Hydrolysis products and corrected R_f - values in system 1-11*

Hydrolysis Product	*Corrected R_f- values in system*										
	1	2	3	4	5	6	7	8	9	10	**11**
ABFB	70	69	74	83	81	93	83	11	71	69	**32**
ABP	64	61	66	85	86	88	77	9	68	69	**2**
ACB	76	69	76	86	90	91	78	12	76	71	**27**
ACFB	76	68	74	86	89	93	78	11	75	70	**31**
ADB	77	70	76	87	89	93	78	11	76	70	**33**
ANB	71	65	68	85	90	91	77	5	69	69	**15**
ANCB	72	66	70	86	87	93	77	4	69	70	**16**
ANFB	71	66	68	86	87	91	77	4	69	70	**16**
CCB	83	74	82	88	92	95	80	58	82	74	**68**
CFMB	71	69	81	83	77	90	75	48	77	69	**55**
CFTB	74	73	80	85	77	93	79	44	77	73	**74**
CPB	75	73	80	86	86	91	83	48	77	72	**57**
MACB	82	72	82	88	89	93	79	53	81	71	**52**
MDB	81	69	81	87	82	93	75	49	81	71	**57**
MNB	66	66	80	83	76	86	73	31	77	68	**29**
MNFB	79	63	80	86	89	90	77	20	79	71	**29**
TCB	81	74	81	89	94	95	82	49	81	75	**74**

* Solvent system 1 = Chloroform-acetone (80:20); 2 = Ethyl acetate, 3 = Chloroform-methanol (90:10); 4 = Ethyl acetate-methanol-conc.ammonia (85:10:5) ; 5 = Methanol; 6 = Methanol-n-butanol (60:40; 0.1 mol/l NaBr); 7 = Methanol-conc.ammonia (100:1.5), 8 = Cyclohexane-toluene-diethylamine (75:15:10); 9 = Chloroform-methanol (90:10); 10 = Acetone; **11 = Toluene.**

Acknowledgements

The authors express their gratitude to the different manufacturers who generously gave their help and provide substances for obtaining these data. Finally, the authors would like to acknowledge the support from many members of the DFG Commission for Clinical-Toxicological Analysis and the TIAFT. The investigations were supported by the Deutsche Forschungsgemeinschaft (DGF).

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