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Modern Screening Strategies in Analytical Toxicology with Special Regard to New Benzodiazepines*

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Summary. Screening procedures for the detection of toxicologically relevant substances have become of ever-increasing importance due to the rapid development of new substances. Identification methods must be simple, sensitive, and practicable. This article describes standardized chromatographical (corrected R_f^c values, retention indices) and immunological methods (enzyme-multiplied immunoassay technique, fluorescent polarization immunoassay) with special regard to the screening of some newer benzodiazepines, a class of substances that is still expanding. Some of these new compounds may be integrated in well-known screening procedures (via aminobenzophenones and detection by the Bratton-Marshall reagent); others require special concepts for detection. The problems are indicated and discussed, including the use of high-pressure-liquid chromatography and mass spectrometry; recommendations are given.

Key words: Screening, benzodiazepines – Benzodiazepines – R_f^c values, benzodiazepines – Retention index benzodiazepines – Immunological methods, benzodiazepines

Zusammenfassung. Die ständig zunehmende Zahl toxikologisch relevanter Fremdstoffe erfordert leicht praktikable aber dennoch empfindliche und aussagekräftige Screeningverfahren, um im Rahmen der General-Unknown-Analyse möglichst rasch gezielte Hinweise auf eine bestimmte Substanz oder eine ganze Wirkstoffgruppe zu erhalten.

Die Senatskommission für Klinisch-toxikologische Analytik der Deutschen Forschungsgemeinschaft hat in Zusammenarbeit mit der TIAFT (The International Association of Forensic Toxicologists) bereits vor längerer Zeit die

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Aufgabe übernommen, solche Screeningkonzepte zu überprüfen, weiter zu entwickeln und verläßliches Datenmaterial zur Verfügung zu stellen.

In der vorliegenden Arbeit werden zunächst die Konzepte des korrigierten R_f^c-Wertes in der Dünnschichtchromatographie, des Retentionsindex in der Gaschromatographie sowie wichtiger immunologischer Verfahren zum Screening beschrieben, um die prinzipielle Arbeitsweise der verschiedenen Methoden zu veranschaulichen. Neben diesen methodischen werden jedoch auch stoffliche Aspekte berücksichtigt: Für Neuentwicklungen auf dem Gebiet der Benzodiazepine, einer immer noch rasch expandierenden Stoffklasse, wurde umfangreiches Datenmaterial erarbeitet und zusammengestellt. Einige dieser Benzodiazepine lassen sich in bestehende Screeningprogramme (z.B. über Benzophenone und Bratton-Marshall-Detektion) integrieren, andere können nur über die R_f^c-Werte erfaßt werden. Schwierigkeiten und Vorschläge für ihre Beseitigung werden auch im Zusammenhang mit den immunologischen Verfahren aufgezeigt bzw. diskutiert. Weiterhin werden einige Aspekte der Hochdruckflüssigkeitschromatographie (HPLC) und der Massenspektrometrie (MS) im Zusammenhang mit Screeningfragen angesprochen. Der Beitrag schließt mit Empfehlungen zum Einsatz der einzelnen Methoden unter besonderer Berücksichtigung des Benzodiazepin-Screenings.

Schlüsselwörter: Screening, Benzodiazepine – R_f^c-Werte, Benzodiazepine – Retentionsindex, Benzodiazepine – Immunologische Methoden, Benzodiazepine

Introduction

Screening procedures – simple, but reliable methods for the recognition of a special compound or class of substance – are becoming more and more important because of the continuously increasing number of toxicologically relevant chemicals (e.g., drugs, pesticides, herbicides, bactericides, household products, cosmetics, or doping agents); the need for forensic- and clinical-toxicological analyses is growing. Therefore, it is one of the major tasks of the Senate Commission for Clinical-Toxicological Analysis of the Deutsche Forschungsgemeinschaft (DFG)[1] to collect, evaluate and develop screening and detection methods for substances frequently encountered in human forms of intoxication. Also, one of the primary aims of the Commitee for Systematic Toxicological Analysis of The International Association of Forensic Toxicologists (TIAFT)[2] is to compile current reliable data to facilitate the detection of drugs, poisons, and other

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toxicologically and forensically relevant substances, particularly those that fall into the category of the "generally unknown." This category is regarded as one of the most difficult analytical challenges.

In the early 1980s, the STA Commitee and the DFG Senate Commission for Clinical-Toxicological Analysis began a cooperative venture, which resulted in several startling publications dealing with screening strategies and exhaustive compilations of reliable and comparable chromatographical data [16, 18, 19].

This article reviews the standardizing steps, which are the base of the published chromatographic data, as well as other analytical methods commonly used as screening approaches for "general unknowns" (e.g., immunological and spectroscopical screening tests).

As practical examples, many benzodiazepines were chosen that have been recently developed and marketed. Without a doubt, this class of substances, which are therapeutically used as tranquilizers, hypnotics, anticonvulsants and muscle relaxants acting on the central nervous system, ranks among the most frequently prescribed drugs; these drugs are regularly encountered in toxicological analysis.

Thin-Layer Chromatography – The Concept of the Corrected R_f^c Value

The reproducibility of the hR_f value[3] is governed by many factors (e.g., activity of the sorbent, state of saturation of the development tank, running distance, amount of drug applied to the chromatogram, geometry of the chamber, temperatur, etc.). With the aim of eliminating some of these parameters, Galanos und Kapoulas [23] and de Zeeuw et al. [81] recommended a multiple correction graph (linear interpolation, which can be carried out graphically or by calculation, using the following equation [19]):

$$R_f^c(p) = \frac{\Delta^c}{\Delta}\left(R_f(p) - R_f(t_n)\right) + R_f^c(t_n)$$

wherein

$$\Delta^c = R_f^c(t_n) - R_f^c(t_{n+1})$$
$$\Delta = R_f(t_n) - R_f(t_{n+1})$$

$R_f^c(p)$: corrected R_f value of the unknown substance p

$R_f(p)$: measured R_f value of the unknown substance p

$R_f^c(t_n)$: corrected R_f value of the reference substance nearest to p (lower value, possible starting point = 0)

$R_f^c(t_{n+1})$: corrected R_f value of the other reference substance nearest to p (higher value, possible solvent front = 100)

Note: $R_f^c(t)$ values are taken from tables (see Table 1, last column)

$R_f(t_n)$ and $R_f(t_{n+1})$: measured R_f values of the reference substances t_n and t_{n+1}, respectively.
Δ^c: Difference of the corrected R_f values of the reference substances, which are situated nearest to the R_f value of the unknown compound p (taken from tables; see Table 1, last column).

[3] $hR_f = \dfrac{\text{Distance from start to substance}}{\text{Distance from start to solvent front}}$

Table 1. Running systems according to [19]; also see this report for more details regarding the discrimination power and identification power (including error windows) of the different solvent systems

Solvent[a]	Adsorbent	Reference compounds[b]	hR_f^c
(1) Chloroform-acetone (80 + 20)	Silica	Paracetamol	15
		Clonazepam	35
		Secobarbital	55
		Methylphenobarbital	70
(2) Ethyl acetate	Silica	Sulfathiazole	20
		Phenacetin	38
		Salicylamide	55
		Secobarbital	68
(3) Chloroform-methanol (90 + 10)	Silica	Hydrochlorothiazide	11
		Sulfafurazole	33
		Phenacetin	52
		Prazepam	72
(4) Ethyl acetate-methanol-concentrated ammonia (85 + 10 + 5)	Silica	Morphine	20
		Codeine	35
		Hydroxyzine	53
		Trimipramine	80
(5) Methanol	Silica	Codeine	20
		Trimipramine	36
		Hydroxyzine	56
		Diazepam	82
(6) Methanol-*n*-butanol (60 + 40); 0.1 mol/l NaBr	Silica	Codeine	22
		Diphenhydramine	48
		Quinine	65
		Diazepam	85
(7) Methanol-concentrated ammonia (100 + 1.5)	Silica impregnated with 0.1 mol/l KOH and dried	Atropine	18
		Codeine	33
		Chlorprothixene	56
		Diazepam	75
(8) Cyclohexane-toluene-diethylamine (75 + 15 + 10)	Silica impregnated with 0.1 mol/l KOH and dried	Codeine	6
		Desipramine	20
		Prazepam	36
		Trimipramine	62
(9) Chloroform-methanol (90 + 10)	Silica impregnated with 0.1 mol/l KOH and dried	Desipramine	11
		Physostigmine	36
		Trimipramine	54
		Lidocaine	71
(10) Acetone	Silica impregnated with 0.1 mol/l KOH and dried	Amitriptyline	15
		Procaine	30
		Papaverine	47
		Cinnarizine	65

[a] Eluent composition: volume + volume; saturated systems are used throughout except for systems 5 and 6, which are used with unsaturated solvent tanks

[b] Solutions of the four reference compounds at a concentration of approximately 2 g/l for each drug

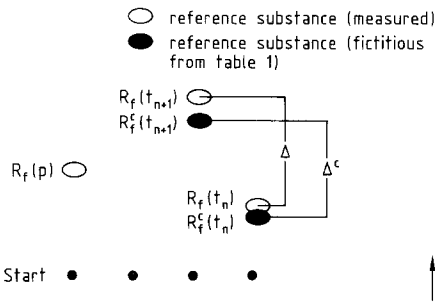

Fig. 1. Calculation of the R_f^c value from measured TLC data ○. The black spots ● (except starting points) indicate fictitious values that must be taken from tables (see Table 1, last column)

$$\Delta^c = R_f^c(t_n) - R_f^c(t_{n+1})$$

Δ: Difference of the measured R_f values of the reference substances, which are situated nearest to the R_f values of the unknown compound p

$$\Delta = R_f(t_n) - R_f(t_{n+1})$$

Figure 1 illustrates the relationships between the different values described above.

Report VII of the DFG Commission for Clinical-Toxicological Analysis (special issue of the TIAFT Bulletin) presents R_f^c data of some 1,100 toxicologically relevant substances (drugs, illicit products, pesticides, metabolites, endogenous compounds) in ten standardized TLC systems compiled in Table 1 [19].

Screening of the Newer Benzodiazepines via R_f^c Values

The R_f^c values of some of the newer benzodiazepins are presented in Tables 2 and 3. Detection can be performed either by ultraviolet visualization (fluorescence excitation or quenching) or by spraying with commonly used [75] base reagents (e.g., Dragendorff reagent or iodoplatinate).

Screening of the Newer Benzodiazepines via Aminobenzophenones and Bratton-Marshall Detection

Thin-layer-chromatography (TLC) is the preferred method for screening benzodiazepines and their metabolites. A further well-known procedure involves hydrolysis to yield aminobenzophenone derivatives, which are then extracted, separated by TLC, and photolytically dealkylated. The products are diazotized and coupled to azo dyes (e.g., with the Bratton-Marshall reagent). This method has already been applied to numerous benzodiazepines [17, 51] and its specificity established [62]. Detailed descriptions of the different steps of the screening procedure are available (see [49] in English and [54] in German, respectively). When using normal 20 x 20 cm TLC plates, 80–160 min must be regarded as the complete analysis time, which can be reduced drastically when modern high-performance-thin-layer chromatography (HPTLC) is applied with special silica gel preparations such as sorbents and 10 x 10 cm plates.

Figure 2 below indicates the positions of the acid-hydrolysis products (aminobenzophenones) of some of the newer benzodiazepines with the classic 2-oxo-structure. The chromatographic conditions were chosen in accordance with

Table 2. Corrected R_f^c values of the nontetracyclic newer benzodiazepines in ten solvent systems according to [19]; see Table 1

Substance	Solvent systems									
	1	2	3	4	5	6	7	8	9	10
Haloxazolam	46	48	65	77	82	90	74	11	66	62
ABFB (hydrolysis product)[c]	70	69	74	83	81	93	83	11	71	69
Ethyl loflazepate	53	58	62	74	85	93	76	0	62	67
N-Desalkyl-2-oxo-quazepam (= *N*-Desmethyl-fludiazepam)	34	42	54	71	85	89	74	4	57	59
3-Hydroxy-*N*-desalkyl-2-oxo-quazepam (= 3-Hydroxy-*N*-desmethyl-fludiazepam)	15	28	35	49	88	89	67	0	30	38
ACFB (hydrolysis product)[c]	69	68	72	81	77	89	74	12	71	68
Cloxazolam	39	45	63	75	78	84	73	0	66	59
Delorazepam	35	41	57	72	82	86	73	5	58	56
ADB (hydrolysis product)[c]	70	69	74	83	81	92	84	11	70	68
Quazepam	78	71	78	83	87	96	74	27	75	76
2-Oxo-quazepam	59	57	70	80	87	90	78	16	69	71
3-Hydroxy-2-oxo-quazepam	42	52	58	69	88	90	71	2	55	59
CFTB (hydrolysis product)[c]	74	73	80	85	77	93	79	44	77	73
Pinazepam	65	61	73	81	85	92	75	29	72	70
CPB (hydrolysis product)[c]	75	73	80	86	86	91	83	48	77	72
Fludiazepam	56	50	67	75	78	86	76	24	69	63
CFMB (hydrolysis product)[c]	71	69	81	83	77	90	75	48	77	69
Nimetazepam	53	46	71	77	81	81	74	12	70	55
MNB (hydrolysis product)[c]	66	66	80	83	76	86	73	31	77	68

[c]For abbreviations, see [57] and Fig. 2 (legend)

proposals of the DFG [17], which are described in detail by Schneider and Schütz [49] .

The R_f^c values of numerous other benzodiazepines, metabolites, and aminobenzophenones are reported in a handbook [55].

In summary, one can state that the corrected R_f^c value is a highly valuable instrument for a better TLC screening.

Error windows for the different solvent systems are based on multiplying the interlaboratory standard deviation of measurement of hR_f values by three; these range from 5 to 11 units and are described and discussed in the DFG report [19].

The influence of different parameters, such as prechromatographic treatment of the TLC plate, different temperatures during development, salts and fat in biological extracts and the effect of multiple use of the solvents, were all

This correction procedure reduces the standard deviations of measurement of R_f-values by about 50% (Moffat [34])

Table 3. Corrected R_f^c values of the tetracyclic newer benzodiazepines in ten solvent systems according to [19]; see Table 1

Substance	Solvent systems									
	1	2	3	4	5	6	7	8	9	10
Adinazolam	9	2	51	57	68	66	69	6	55	22
Mono-*N*-demethyl-adinazolam	1	0	28	40	49	39	63	1	36	2
Alprazolam	7	2	40	49	70	66	67	1	57	14
α-Hydroxyalprazolam	4	4	37	37	76	76	72	1	44	18
4-Hydroxyalprazolam	3	2	26	26	73	69	67	1	25	7
Estazolam	7	5	43	50	71	60	71	2	53	25
Triazolam	5	2	41	43	69	65	69	1	40	16
α-Hydroxytriazolam	4	3	39	42	75	74	71	4	49	21
4-Hydroxytriazolam	3	1	29	24	75	71	63	0	29	8
Brotizolam	15	4	53	54	74	71	72	5	52	27
α-Hydroxybrotizolam	7	6	41	45	78	78	72	2	46	31
4-Hydroxybrotizolam	5	4	37	28	76	76	68	1	35	13
Loprazolam	3	1	36	35	24	15	40	1	48	5
Midazolam	13	5	53	64	71	70	72	6	60	19
α-Hydroxymidazolam	3	4	41	53	73	72	70	3	52	8
4-Hydroxymidazolam	5	5	37	49	76	77	74	1	43	15
α,4-Dihydroxymidazolam	2	5	33	38	78	81	75	4	25	14

investigated by Bogusz et al. [8, 10, 11, 12] and by Borchert et al. [13]. It is the result of the work of Bogusz et al. that co-extracted materials generally decrease R_f values and increase their standard deviations, resulting in larger error windows. Nevertheless, the concept of the corrected. R_f^c value has proven its enormous pragmatic usefulness.

Other aspects of TLC-screening [e.g., in situ registration of UV spectra, highly-sensitive fluorescence detection after derivatization (pre- and after-run modes)] are currently being investigated and will be the subject of forthcoming publications [22]. For additional information, see other publications [15, 20, 25, 34, 36, 37, 39–41, 50, 56, 65, 76].

Gas Chromatography – the Concept of the Retention Index

Gas Chromatography is one of the most useful and frequently applied tools for the screening and identification of organic compounds (preferably in combination with spectroscopic methods, e.g., mass-spectrometry, and recently infrared spectroscopy in the FTIR mode). The primary measuring parameter is the retention time, which is governed by many variables, such as composition of the stationary phase, column length, flow of the carrier gas, oven temperature and other variables. Therefore, the retention times are hardly appropriate to serve as reliable and intercomparable data. Moderate improvement can be seen in measurement of the relative retention time, but the most useful instrument is

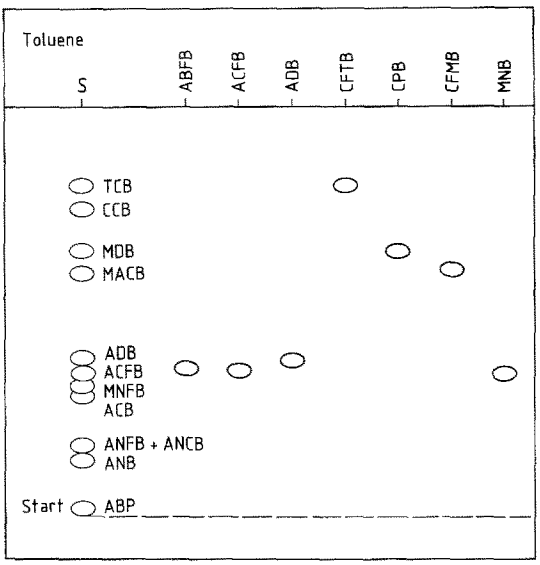

Fig. 2. Positions of the acid hydrolysis products (aminobenzophenones) of some newer benzodiazepines (solvent:toluene; sorbent:silica gel 60 F_{254}; see [17] and [49] for more details). *Abbreviations: ABFB* 2-amino-5-bromo-2′-fluoro-benzophenone (from haloxazolam); *ACFB* 2-amino-5-chloro-2′-fluorobenzophenone (from ethyl loflazepate, *N*-desalkyl-2-oxoquazepam, *N*-desmethyl-fludiazepam, and other metabolites); *ADB* 2-amino-2′, 5-dichlorobenzophenone (from cloxazolam, delorazepam, mexazolam, and lorazepam); *CFTB* 5-chloro-2′-fluoro-2-(2,2,2-trifluoroethyl-amino) benzophenone (from quazepam, 2-oxoquazepam and 3-hydroxy-2-oxoquazepam); *CPB* 5-chloro-2-(2-propinyl) benzophenone (from pinazepam and 3-hydroxy-pinazepam); *CFMB* 5-chloro-2′-fluoro-2-methylaminobenzophenone (from fludiazepam and 3-hydroxy-fludiazepam); *MNB* 2-methylamino-5-nitrobenzophenone (from nimetazepam). *Remarks:* Abbreviations concerning the hydrolysis products of long-known benzodiazepines are listed in [17, 50, 52, 55, 57]. Also see [55] for the structural formulae of the newer benzodiazepines

without any doubt the *retention index* (RI)[4] developed and introduced by Kovats [29] .

[4]The retention index RI(A) can be calculated by using one of two essentially identical equations [18]:

$$\mathrm{RI(A)} = 100\,(y-x)\frac{\log \frac{t(\mathrm{A})}{t(\mathrm{X})}}{\log \frac{t(\mathrm{Y})}{t(\mathrm{X})}} + 100x \tag{1}$$

$$\mathrm{RI(A)} = [\mathrm{RI(Y)} - \mathrm{RI(X)}]\frac{\log \frac{t(\mathrm{A})}{t(\mathrm{X})}}{\log \frac{t(\mathrm{Y})}{t(\mathrm{X})}} + \mathrm{RI(X)} \tag{2}$$

t(A) = net retention time of a substance A
t(X) = net retention time of the *n*-alkane C_xH_{2x+2} eluting immediately before A
t(Y) = net retention time of the *n*-alkane C_yH_{2y+2} eluting immediately after A
x = carbon number of the *n*-alkane C_xH_{2x+2}
y = carbon number of the *n*-alkane C_yH_{2y+2}
RI(A), RI(X), etc. = retention indices of substances A, X, etc.

The retention index RI(A) can also be obtained from a simple graph [18].

The interlaboratory standard deviation of the RI is in the order of 15–20 RI units (Moffat [38]; Berninger and Möller [7]. According to DFG [18], a "search window" of ± 50–60 RI units should be taken into consideration when working under temperature-programmed conditions with an almost linear relationship between the carbon number of the *n*-alkanes and the retention time (Peel and Perrigo [43, 44]). The search window mentioned above will also take the temperature dependency of the RI into consideration. The quality of the column must be tested as described in [18] before starting the analysis.

The RI values of many benzodiazepines are listed [18, 55]. Additional RI indices of newer benzodiazepines on OV-1 are given in Table 4. For screening strategies based on a second stationary phase (e.g., OV-17) see Post [46].

In conclusion, one can say that the introduction and use of the retention index has raised gas chromatography to a higher level with excellent inter laboratory reproducibility. Based on a compilation of Ardrey and Moffat [2], the DFG/TIAFT publications present data on about 1,600 substances in a second revised and enlarged edition [18] , which is succesfully used to solve screening problems all over the world. Use of the retention index within capillary GLC is the subject of broad investigations (DFG commission). For additional information, see [15, 35, 47, 71, 79] and for capillary GLC [9, 80].

Table 4. Retention indices (RI values) of newer benzodiazepines on OV 1 (see text for additional details). This table also presents the UV maxima in different solutions

Substance	Retention index OV-1/ SE-30	UV Maxima		
		EtOH	HCl*	NaOH*
Adinazolam	3060	–	263	–
Mono-*N*-demethyladinazolam	3130	222	270	253
Alprazolam	3050	222	264	258
α-Hydroxy-alprazolam	3010	220	280	–
4-Hydroxy-alprazolam	nm	222	222	222
Brotizolam	3145	240	254	241
α-Hydroxy-brotizolam	nm	243	256	245
4-Hydroxy-brotizolam	nm	241	244	241
Cloxazolam	2405	–	–	–
Delorazepam	2650	320	238/286	227/345
Estazolam	2955	222	278	218
Ethyl-loflazepate	2195	230/320	232	354
Fludiazepam	2460	229	240/282	229
2-Methylamino-5-chloro-2'-fluoro-benzophenone (CFMB)	2133	409	417	417
Flumazenil (Ro-15-1788)	2560	246	–	–
Halazepam	2335	225	233/285	248
2(2,2,2-Trifluoroethyl)-amino-5-chloro-benzophenone (TCB)	2000	386	286/417	400
Haloxazolam	2620	247	242	–

Table 4 (continued)

Substance	Retention index OV-1/ SE-30	UV Maxima		
		EtOH	HCl*	NaOH*
2-Amino-5-bromo-2′-fluoro-benzophenone (ABFB)	nm	–	–	–
Loprazolam	nm	330	329	309
Metaclazepam	2690	370	251/460	370
N-Desmethyl-metaclazepam	2720	444	249/445	362
Bis-Desalkyl-metaclazepam	2850	377	249/444	380
Midazolam	2620	217	212	216
gα-Hydroxy-midazolam	2825	215	215/258	–
4-Hydroxy-midazolam	2580	215	215/251	257
α,4-Dihydroxy-midazolam	nm	217	218	225
Nimetazepam	2730	220/260	282	248/395
2-Methylamino-5-nitro-benzophenone (MNB)	2500	239/367	243/383	383
Oxazolam	2590	247	237	247
Pinazepam	2580	227	238/284/356	–
5-Chloro-2-(2-propinyl)benzophenone (CPB)	2270	397	403	429
Quazepam	2485	286	273	–
2-Oxoquazepam	2270	226	283	231
N-Desalkyl-2-oxoquazepam	2475	228/320	238/282/366	232
3-Hydroxy-*N*-desalkyl-2-oxoquazepam	2300	230	232	215
3-Hydroxy-2-oxoquazepam	2405	227	228	226
2-(2,2,2-Trifluoroethyl)-5-chloro-2′-fluorobenzophenone (CFTB)	2395	232/391	405	405
Triazolam	3130	222	–	–
α-Hydroxy-triazolam	3020	221	218	223
4-Hydroxy-triazolam	nm	221	222	221
Flutazolam	2310	250	254	254
Mexazolam	2670	247	292	248
Etizolam	3090	254	295	254
Tofisopam	3035	312	369	345

*0.1 mol/l
nm = Not measurable without derivatization

High-Pressure Liquid Chromatography (HPLC)

High-pressure liquid chromatography is a good, practicable tool for the quantitative determination of organic and inorganic (ion chromatography) compounds. Its value as a screening method is controversial, as the reproducibility of the retention data cannot be compared with that for gaschromatography. This is caused by enhanced discrimination power and the difficulties connected with the standardization of the stationary phases, mobile phases, and certain

apparatus parameters. Another problem is the development of a suitable reference system (comparable to n-alkanes in gaschromatography). Baker and Ma [5] tested homologous 2-ketoalkanes and Smith et al. [73, 74] alkyl-arylketones, but the reference system is not yet finished.

On the other hand, the combination of HPLC with diode-array detection (DAD) must be considered a highly effective screening method. Therefore, the principal absorption data of newer benzodiazepines and major metabolites are presented in Table 4. For the complete spectra graphs of the spectra, see Schütz [55]. Additional information is available in the literature [6, 15, 26–28, 72] and in an open letter to TIAFT members on the measurement of retention values in HPLC which was recently published by Moffat (1988, TIAFT Bulletin).

Immunological Methods

Immunoassays have a firm place among routine methods for the screening of drugs in biological fluids and should be used as confirmation tests for chromatographic screening procedures (the reverse is also practiced in many laboratories).

The tests can be subdivided into two broad classes:

Homogenous Assays (no separation step required): Enzyme Multiplied Immunoassay Technique (Emit); fluorescent polarization immunoassay (FPIA); substrate-labeled fluorescence immunoassay (SLFIA); rate nephelometric inhibition immunoassay; apoenzyme reactivation immunoassay system (ARIS); others

Heterogenous Assays (separation step required): Radioimmunoassay (RIA); fluorescence immunoassay; radioreceptor assay (RRA); competitive protein-binding assay (similar to RIA); Luminescence immunoassay (LIA, similar to RIA) in the SPALT (solid-phase antigen luminescence technique) mode[5]; a newly developed, competitive, heterogenous, solid-phase enzyme immunoassay (EIA) described by Bäumler [4].

The scope of this review is to describe and discuss chiefly two commonly used, popular, competitive binding immunoassays for benzodiazepines: the Enzyme Multiplied Immunoassay Technique (EMIT) and the fluorescent polarization immunoassay (FPIA). Precise and informative descriptions of the principles of these tests have recently been given by Maes[6].

Enzyme-Multiplied Immunoassay Technique (EMIT): The principle of EMIT is familiar. In brief, when serum-labeled hapten (drug covalently bound to an enzyme), substrate, and appropriate antibody are mixed, enzyme-labeled hapten and drug compete for binding to the antibody. Since binding to the antibody reduces enzyme activity, the concentration of the drug in the sample is directly related to free hapten concentration and, thus, to enzyme activity. Substrate depletion or product formation is measured spectrophotometrically (also see [25]).

Fluorescent-Polarization Immunoassay (FPIA): The recent application of fluorescent polarization to the analysis of drugs is a particularly innovative approach to homogenous nonisotopic immunoassays. Quantification of antibody-antigen reactions with fluorescent polarization is not new. However, appropriate commercial instruments and reagents, specifically developed for the immunoassay of drugs, have recently become available and appear to have rapidly gained wide acceptance.

[5] M. Möller (Homburg) et al. (presented on the occasion of the annual meeting of the American Association of Forensic Sciences, San Diego, California, 19 February 1987)

[6] Sessions of the DFG Task Group "Analytical-technical developments" (Chairmen: H. Brandenberger, Zürich; T.Daldrup, Düsseldorf) held at Basel and München

Briefly, if a fluorophore is excited by polarized light, its emission will be similarly polarized. Rotation of the molecule results in a reduced degree of polarization or an increase in the randomness of the light emitted. A larger molecule with slower rotation (a longer rotational relaxation time) will emit a greater proportion of polarized light. According to this principle, fluorescein-labeled hapten, antibody, and sample are mixed and, after a fixed time, the intensity of the emitted polarized light is measured. Binding of the labeled hapten to antibody results in a macromolecule that shows increased fluorescent polarization.

Since this is a competitive binding assay, the concentration of antibody-bound label and, consequently, fluorescent polarization are inversely related to the concentration of unlabeled drug in the sample.

The commercial system[7] includes a completely integrated and automated fluorescence polarization instrument and sample-processing system, which dilutes the samples, adds reagents, measures the fluorescence polarization signal, corrects for background and random fluorescence, and calculates drug concentration.

Other than the loading of the multisample carousel, no sample handling is necessary, with the exception of the digoxin assay, which requires an offline protein precipitation step. No enzyme or substrate is required, and reproducibility appears to be excellent. Analysis of a single sample can be completed in about 20 min and of a carousel of 20 samples in 40 min.

The marketed fluorescence polarization assay utilizes a dedicated instrument, which cannot be easily adapted to other laboratory applications, it may therefore be a less cost-effective option for the small laboratory that handles relatively few samples[8]. Despite the elegance of the instruments, the accuracy of the assay still depends on the specificity of each batch of antibodies prepared by the supplier. Therefore, the calibration values should be kept under regular check.

The importance of the TDx test is illustrated by the fact that in 1986 about 80% of immunological screenings in The Netherlands were carried out with this system (D.R.A. Uges, personal communication).

Problems with immunological Benzodiazepine Tests: False-negative immunological screening results, after the application of bromazepam and flunitrazepam, were described by Bäumler [3]. Müller-Oerlinghausen (personal communication) observed no positive findings (morning urine) when oxazepam was administrated in single or repeated doses of 10 to 20 mg the evening before. In our laboratory discrepant findings between thin-layer chromatographical and enzyme-immunological (EMIT) tests were regularly observed after normal therapeutic doses of bromazepam, flunitrazepam, lorazepam, oxazepam (TLC-positive/EMIT-st negative) and triazolam (TLC-negative/EMIT-st slightly positive). Also see Oellerich [42].

These discrepancies may be caused by: the formation of conjugates (e.g., lorazepam-glucuronide is not detectable by EMIT-st); low concentrations (e.g., lower than cut-off levels); poor cross-reactivities (e.g., bromazepam); no formation of diazotable benzophenones (e.g., tetracyclic benzodiazepines such as triazolam and others).

Special Investigations with Immunoassays for Benzodiazepines: Both immunoassays (EMIT-st and TDx) have revealed unexpectedly good cross-reactivities with the new class of tetracyclic benzodiazepines (e.g., TDx: 116% alprazolam and 83% triazolam when compared with 100% N-desmethyldiazepam.

[7] TDx system

[8] A new ADx system has been especially developed for laboratories with few samples (unit dose mode) and will be presented in the near future (summer 1988)

The threshold ranges and detection limits for the immunological screening of many tetracyclic benzodiazepines (adinazolam, alprazolam, brotizolam, estazolam, loprazolam, midazolam, triazolam and major metabolites) using the EMIT and TDx systems have recently been reported in this journal [68]. In most cases, the cross-reactivities of the two systems are comparable, but there are also remarkable differences. The detection limits are mainly in the range between 0.2 and 0.5 mg/l but in some cases limits are also considerably higher.

In many cases, the usual assay cut-off level for benzodiazepines (200 ng/ml) can be decreased to 50 ng/ml (von Meyer, personal communication), resulting in a higher sensitity and a smaller number of discrepant findings when compared with the highly-sensitive detection method of Bratton and Marshall (TLC screening) [14, 19].

The precision of the TDx test yields consistently low coefficients of variation [78]:

	200 ng/ml	300 ng/ml	1,000 ng/ml
Mean value (ng/ml)	196.00	293.00	1,020.00
CV within run (%)	2.57	2.00	1.36
CV between run (%)	4.11	2.61	1.99

These values are in good accordance with our own results published in 1987 [68].

An exhaustive compilation of cross-reactivity data concerning compounds other than benzodiazepines has also been published [78]. Cross-reactivities of up to 0.8% with the TDx benzodiazepines assay were observed in only 17 of 176 substances tested (cocaine, dimetacrine, diphenhydramine, doxylamine, fenoprofen, flufenamic acid, indometacin, iprindole, mefenamic acid, methadone, methotrimeprazine, orphenadrine, phencyclidine, phendimetrazine, phenylbutazone, phenytoin and trazodone).

In intensive conjugation (e.g., 3- or 4-hydroxybenzodiazepines), cleavage of the conjugates by enzymatic hydrolysis prior to analysis as described by Schütz et al. [69] can lead to better results. In other cases of very low concentrations (e.g., flunitrazepam, lorazepam and tetracyclic benzodiazepines after single therapeutic doses) and/or poor cross-reactivities (e.g., bromazepam, chlordiazepoxide, demoxepam), an enrichment procedure using Extrelut or some other solid-phase extraction system prior to enzymatic screening tests can also help to avoid "false-negative" results as demonstrated in Schütz et al. [69] and in Fig. 3.

Mass Spectrometry

This review will not be complete without a few lines dealing with this modern and highly sophisticated analytical method, which can also be used as screening procedure. The mass spectrometer is without a doubt the most specific detector of gas (or high-pressure liquid) chromatography, and the publications referring to this method are myriad. Some collections of mass spectra should be mentioned, e.g., those by Ardrey et al. [1], Maurer und Pfleger [30, 31], Pfleger et al. [45], Mills et al. [32], Mills et al. [33], and Schütz [51].

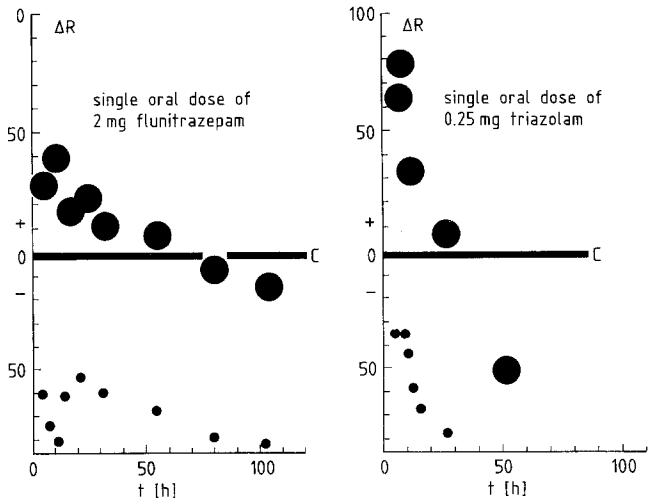

Fig. 3. Screening findings with EMIT-st. *C* Cut-off level (threshold); • direct screening (without enrichment); ● enrichment via Extrelut (see [69] for details)

So far the FTIR mode has not been regarded as suitable for the screening of benzodiazepines. Thus, this new method still needs final evaluation and should not be considered for the time being. A forthcoming title [55] presents the spectra (GLC-MS, LC- MS, UV, IR) of the new benzodiazepines described in this article and many metabolites and hydrolysis products. See Suzuki et al. [77] for mass spectra of benzophenones and Schütz et al. [70] for infrared spectroscopy.

Discussions and Recommendations[9]

Thin-Layer Chromatography (corrected R_f^c values): This universally applicable procedure can be recommended for the screening of benzodiazepines, metabolites, and hydrolysis products, as well as many other drugs and poisons that are not benzodiazepines. The R_f^c value concept is the method of choice for the general screening approach. Because of the lack of tetracyclic benzodiazepines (e.g, adinazolam, alprazolam, brotizolam, estazolam, loprazolam, midazolam, and triazolam) to yield primary aromatic amines by acid hydrolysis, the R_f^c values and less specific detection reagents (e.g., Dragendorff reagent or iodoplatinate) are the only practicable TLC screening procedures for this class of benzodiazepines.

Thin-layer Chromatography (Bratton-Marshall detection): This well known and documented screening procedure via acid hydrolysis, photolytical dealkylation, diazotization and coupling with Bratton-Marshall reagent to yield azo dyes [17] is applicable to the new benzodiazepines haloxazolam (via ABFB), ethyl

[9]For publications dealing with the analytical data of a single benzodiazepine see: alprazolam [67], brotizolam [66], clotiazepam [60], halazepam [61], loprazolam [64], midazolam [53], pinazepam [63], tetrazepam [58], triazolam [59]

loflazepate and quazepam metabolites (via ACFB), cloxazolam, mexazolam and delorazepam (via ADB), quazepam und metabolites (via CFTB), pinazepam (via CPB), fludiazepam (via CFMB) and nimetazepam (via MNB). Because of its higher specificity and sensitivity, Bratton-Marshall detection should be preferred as a screening method when these benzodiazepines are encountered.

Gas Chromatography (Retention Index): The concept of the retention index (RI) is recommended as another tool for the general screening of benzodiazepines and non benzodiazepines. If only benzodiazepines are screened, the ECD detector is a highly sensitive detection system; otherwise the NPD detector indicates most of the common drugs and permits discrimination of matrix substances. It must be remembered that some polar benzodiazepines and metabolites (e.g., loprazolam, hydroxy metabolites of tetracyclic benzodiazepines) cannot be separated on OV-1 (as a commonly used stationary phase) without derivatization prior to GLC. Beyond it, the cleavage of conjugates is unavoidable.

Immunological Methods (EMIT, FPIA (TDx)): The immunoassays mentioned above are good systems for the rapid and reliable screening of large numbers of samples, especially in cases of multiple doses and single overdoses. False-negative findings must be taken into account after the application of single doses of *small amounts* of benzodiazepines (e.g., flunitrazepam, lorazepam, tetracyclic benzodiazepines), 3-hydroxylated benzodiazepines (lorazepam, oxazepam, lormetazepam) and benzodiazepines with poor cross-reactivities (e.g., chlordiazepoxide, bromazepam). Discrepant findings between TLC and immunological screening methods may be avoided by additional pre screening procedures, such as enzymatic cleavage of conjugates, decreasing the cut-off level of TDx by modification of the software and/or enrichment techniques (Extrelut).

It is remarkable that the newly developed tetracyclic benzodiazepines can be screened very well by both methods tested (limit generally 0.2–0.5 mg/l) due to their unexpectedly good cross-reactivities. The TDx test was revealed to be also a good quantitative method with outstanding coefficients of variation. It must be remembered, however, that the printed results (expressed as ng/ml) indicate "ng benzodiazepine *equivalents*/ml" with regard to the calibration performed with *N*-desmethyldiazepam.

Mass Spectrometry (MS): Mass spectral confirmation of the results obtained by chromatographic or immunological screening tests is without a doubt the most reliable strategy and is unavoidable, especially in the forensic sector of analytical toxicology. Modern systems, such as ion trap detectors (ITD) and mass selective detectors (MSD) can be set up as facilities at a reasonable cost.

The retrieval of analytical data with the assistance of computer search systems can easily be the subject of another exhaustive review. Some basic publications should be mentioned, however: eight-peak index of mass spectra by Ardrey et al. [1]; the mean list-length approach by Franke et al. [21, 48]; a computer search system using TLC, GLC and UV data by Gill et al. [24]; computerized

GLC/MS by Maurer and Pfleger [30, 31]; the generally accessible data bank in STA by de Zeeuw et al. [81].

A combined screening strategy, which is based on the R_f^c values in systems 4 and 5 (see Table 1), the retention index on OV-1 and the UV absorption (in ethanol, 0.1 m HCl and 0.1 *m* NaOH) has proven its usefulness in our laboratory. The DFG/TIAFT group should also be encouraged to publish materials (e.g. floppy disks) for the computer-aided evaluation of the data compiled in [18] and [19].[10]

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