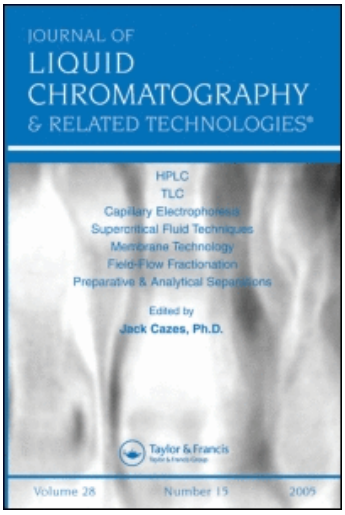

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INTERLABORATORY APPLICABILITY OF A RETENTION INDEX LIBRARY OF DRUGS FOR SCREENING BY REVERSED PHASE HPLC IN SYSTEMATIC TOXICOLOGICAL ANALYSIS

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ABSTRACT

The retention indices (RI) of 47 selected acidic, neutral and basic drugs were determined on 7 reversed-phase (octyl- and octadecylsilica) columns in two laboratories in the 1-nitroalkane scale, using either 1-nitroalkane homologues or selected drugs, whose RI values were previously determined on the reference column. Obtained values were compared with the library values, determined previously on the reference column. Retention indices, calculated with drugs as RI markers, showed distinctly lower deviations from the library values and lower inter-column variability: The mean standard deviation of RI for all drugs analyzed on all columns in the 1-nitroalkane scale was 44.3 RI units, against 10.3 units when selected drugs were used as RI markers. The deviations from the listed values, calculated for each column separately with drugs as markers, were in 95% of cases smaller than 20 RI units, and in 80 % smaller than 10 units. The largest differences between the experimental and listed values were observed for column that differed in type of silica support, type of stationary phase (C8 versus C18) or column length in comparison to the reference column. The kind of silica support contributed more to the variability than the type of stationary phase. The results indicate that it is possible to use and exchange HPLC data based on a retention index library.

INTRODUCTION

High-pressure liquid chromatography (HPLC) can be a potentially useful method in toxicological screening: its identification power may be of the same order as that of gas chromatography and is not hampered by the volatility of the substances under investigation. Moreover, the use of a diode-array detector (DAD) can provide a UV spectrum of a separated substance as second powerful identification parameter, besides the retention time. Both straight phase (1-3) and reversed-phase silica column packings (4-13) have been applied for screening purposes in toxicology. The main drawback of the data bases generated in these studies is that the retention parameters given for substances (retention times or capacity factors) are system-dependent and that, therefore, the data are not transferable to the other laboratories.

It has been repetitively stated, that the selectivity of different commercial brands of the same type of column packing, e.g. octadecylsilica, may differ largely, due to differences in kind of silica support and in coating procedures (14-18), what makes the primary retention parameters - retention times or capacity factors - absolutely incomparable. Particularly affected were basic substances, which may react with free silanol groups on the silica surface (16,19). The variability in the mobile phase composition, its pH, ionic strength, temperature or instrumentation may also greatly influence the reproducibility of retention parameters. In extreme cases, the elution order of substances analyzed on commercially different ODS-phases may change (20-23).

All above-mentioned studies have demonstrated, that it is impossible to achieve a sufficiently high level of interlaboratory reproducibility of primary retention parameters, which is required for establishing and using of an interlaboratory database. As an obvious response to these limitations secondary parameters of retention were introduced, i.e. parameters related to selected retention standards. The concept of retention indices (RI), used widely in GC (24,25), was adapted to reversed-phase HPLC (26). Three RI systems were proposed in toxicological analysis: alkane-2-ones (27), alkyl aryl ketones (28) and 1-nitroalkanes (29). The standardization with retention indices have improved the reproducibility of retention data to some extent, but the differences in RI values of drugs observed for different brands of ODS-columns were still unacceptably large. This appeared to be due to the fact that the substances chosen as retention index standards (homologues) did not adequately mimic the chromatographic behavior of the examined drugs of toxicological interest. As a next step in the standardization the homologues were substituted with selected drugs RI markers. These marker drugs had

previously been given their retention index by analyzing them severalfold against a homologous series (e.g. the 1-nitroalkanes). This approach had already been introduced successfully in gas chromatography (30), whereas in thin-layer chromatography the concept of standardization using toxicologically relevant drugs is also preferred (31). Our previous investigations have shown that the use of drugs standards may distinctly reduce the variability of RI values caused by use of different column packings (32-34). The elution conditions and mobile phase composition should be strictly defined and followed, in order to obtain comparable results (35). We have also demonstrated, that the retention behavior of acidic/neutral and basic drugs, expressed as their RI values, is different (35, 36). Therefore, separate sets of drug standards (acidic/neutral and basic) was required for determination of RI-values on different ODS-columns. This approach should be regarded as a limitation of the screening procedure. However, in the toxicological casework the drugs are extracted from the biosamples into separate acidic/neutral and basic fractions. This enables the application of the proper set of drug standards as retention index markers.

In our previous study we developed a library of 225 substances, using a gradient elution system and a retention index scale based on 1-nitroalkanes (37). We have selected 8 acidic and 10 basic drugs as retention index markers for tentative interlaboratory use. The purpose of the present paper was to check the applicability of the library in interlaboratory use, using different instrumentation and different ODS-silica columns. In order to clearly distinguish between retention indices determined by other means than straight chain alkanes as proposed by Kovats (24) and for which the abbreviation RI has become generally accepted, we propose to use RI_{NO2} for indices determined by using 1-nitroalkanes as references and RI^{D}_{NO2} for indices determined by using drug markers whose retention indices were previously determined against 1-nitroalkanes. This would be in line with the recommendations of the Committee for Systematic Toxicological Analysis of The International Association of Forensic Toxicologists.

MATERIAL AND METHODS

25 acidic/neutral and 22 basic drugs, obtained from different manufacturers, were dissolved in methanol/$H_2$0 (1:1) to the concentration of 0.1 $\mu g/\mu l$. They covered several pharmacological classes and eluted throughout the whole range (1-35 min) as observed previously (37). 1-nitroalkane homologues C-1 to C-6 were purchased from Fluka AG

(Buchs, Switzerland). The homologues C-7 and C-8 were synthesized as described elsewhere (38).

Triethylammonium phosphate buffer, pH 3.0 was purchased from Fluka AG, and acetonitrile (gradient grade) was from E.Merck (Darmstadt, Germany).

Seven columns were selected for the study. In the Institute of Forensic Medicine in Aachen the following columns were used:

SUPERSPHER 100 RP-18, 125 x 4 mm, grain 4 μm, fully endcapped (E.Merck, Darmstadt, Germany)

LICHROSPHER 60 RP-Select B, 125 x 4 mm, grain 5 μm (E.Merck, Darmstadt, Germany)

NUCLEOSIL 100-5 C18 AB, 125 x 4 mm, grain 5 μm (Macherey Nagel GmbH, Düren, Germany).

In the University Centre for Pharmacy in Groningen the following columns were examined:

INERTSIL ODS-2, 125 x 4.6 mm, grain 5 μm (GL Sciences Inc., Tokyo, Japan, distributed by VDS Optilab, Berlin, Germany)

ENCAPHARM RP18, 120 x 4.6 mm, grain 5 μm (Dr.Molnar, Berlin, Germany)

LICHROSPHER 100 RP-18, 125 x 4 mm, grain 5 μm (E.Merck, Darmstadt, Germany)

SYNCHROPAK RP-SCD, 100 x 4.6 mm, grain 5 μm (Synchrom Inc., Lafayette, Indiana, USA)

According to the manufacturers' statements, these columns had been specially deactivated to minimize the silanol effects. In our previous study we have demonstrated, that the columns are applicable for analysis of acidic, neutral and basic drugs using the acidic mobile phase described below, with or without amine modifier (38).

The experiments in Aachen were performed using low-pressure gradient system from E.Merck (655-A Pump, L-5000 Controller and AS 2000 A Autosampler) and Type 990 diode array detector (Waters GmbH, Eschborn, Germany).

In Groningen a high-pressure gradient system was used, consisting of two Model 2350 pumps and a Chemsearch data management system (ISCO, Lincoln, Nebraska, USA), a Marathon autosampler (Spark Holland, Emmen, The Netherlands) and a SPD-6A UV detector (Shimadzu, Kyoto, Japan).

The elution conditions were identical as in our previous paper on establishing a HPLC database (37). Gradient elution in acetonitrile-triethylammonium phosphate buffer 25 mM, pH 3.0 was applied with following profile: 0-70 % acetonitrile in 30 min, 5 min at 70% acetonitrile. The flow rate of the mobile phase was 1 ml/min for all columns.

In the case of the Synchropak column, which was shorter, a flow rate of 0.8 ml/min was also applied. The equilibration time between consecutive samples was 10 min, injection volume was 10 μl, temperature ambient (21-23 °C).

All determinations were performed in duplicate, on different days. The retention indices of substances were calculated from their retention times by linear interpolation either between consecutive homologues (nitromethane to 1-nitrooctane, RI-values 100-800) or between marker drugs, whose retention indices had been previously determined in the 1-nitroalkane scale on the reference Superspher RP-18 column (37). Table 1 shows the composition and RI-values of two mixtures of drugs, used separately for acidic/neutral and for basic substances. The 1-nitroalkane mixture and standard drug mixtures were co-analyzed with each series of determinations, and the actual calibration curves were used for calculation of RI-values.

RESULTS AND DISCUSSION

Day-to-day variabilities of retention times of reference substances was negligible, which was in agreement with our previous findings (37). The differences between the replicate determinations of RI-values of drugs, determined in 1-nitroalkane or drug

TABLE 1. DRUG MIXTURES USED AS RETENTION INDEX MARKERS

ACIDIC MIXTURE		BASIC MIXTURE	
NAME	RI_{NO2}	NAME	RI_{NO2}
paracetamol	234	morphine	198
barbital	287	chloroquine	265
brallobarbital	359	benzoylecgonine	295
pentobarbital	405	cocaine	336
secobarbital	437	diphenhydramine	385
clobazam	484	haloperidol	409
indomethacine	610	amitriptyline	446
prazepam	648	thioridazine	504
		meclozine	601
		amiodarone	762

scales, did not exceeded ± 5 RI units. Figures 1 and 2 show the calibration graphs for each column. Except in the case of the Superspher column did the calibration curves for 1-nitroalkanes, acidic and basic drugs have similar shapes. Yet, for the other columns slight to marked differences in the curves could be noted.

The selection of the examined columns allows to identify several parameters, which potentially affect the comparability of results. The listed (library) values were obtained with a Superspher RP-18 column from E.Merck. In the examined set of columns, one was of the same kind and brand as this reference column, but of different batch number (Superspher), four were of the same kind (octadecyl) but of different brands (Nucleosil C-18, EncaPharm RP18, Inertsil ODS-2, Lichrospher RP18) and two were of different kind (Lichrospher Select B, RP-8, and Synchropak RP-SCD). SCD stands for short-chain deactivated, which probably means octylsilane. Furthermore, three of the above-mentioned columns were supplied by the same manufacturer (Superspher, Lichrospher RP18 and Lichrospher Select B - from E.Merck) and were prepared from the same or similar silica support.

Tables 2 and 3 show the retention index values of all 47 drugs, calculated in the 1-nitroalkane scale and with reference drug markers, respectively. In the two last columns the mean RI-values and standard deviations for each substance are shown. Some data values are missing in the tables. It concern drugs which were not available in the laboratory involved during the study.

The RI_{NO2}-values of individual drugs determined in the 1-nitroalkane scale showed deviations from the listed values, ranging from -118 to +79 RI units. Also, the variation in RI-values between the columns were large, with a mean SD value of 44.4 RI units.

The RI^{D}_{NO2}-values of drugs determined in the drug scales differed less than ±10 units in 80% of the cases of the listed values, and less than ±20 units in 95% from the listed values. The mean SD value calculated for all drugs and all columns was 10.3 RI units.

The mean deviations from the listed RI-values of drugs were also calculated for each column separately. The mean values ± SD of these deviations are shown in Table 4. Again, a striking improvement in accuracy (expressed as mean deviation) and precision (expressed as SD of the mean deviation) was observed when the drug mixtures were used as RI markers. Then, very consistent results were obtained for Superspher, Lichrospher RP18 and Lichrospher Select B columns, which are supplied by the same manufacturer and which have similar silica support material. A slightly lower accura-

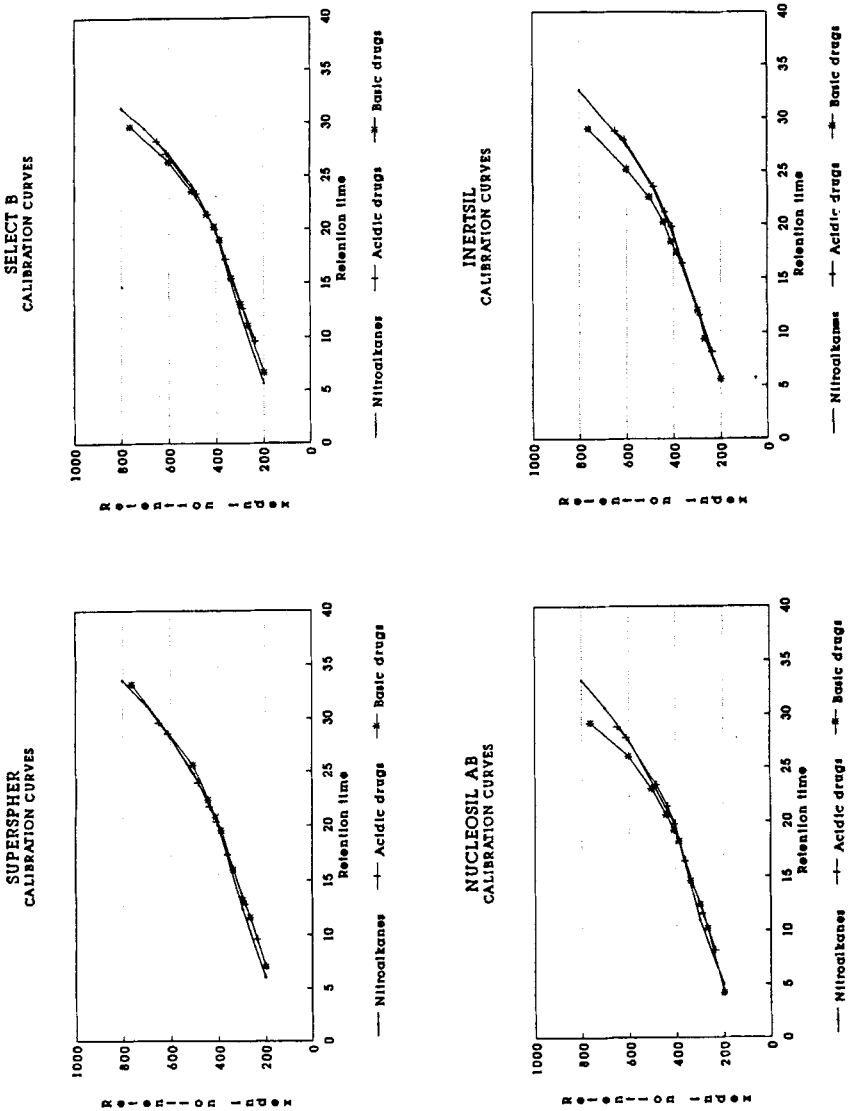

Fig. 1. Calibration curves, prepared with 1-nitroalkanes, acidic and basic drug mixtures for Superspher RP 18, Lichrospher Select B RP 8, Nucleosil C18 AB, and for Inertsil ODS-2 columns.

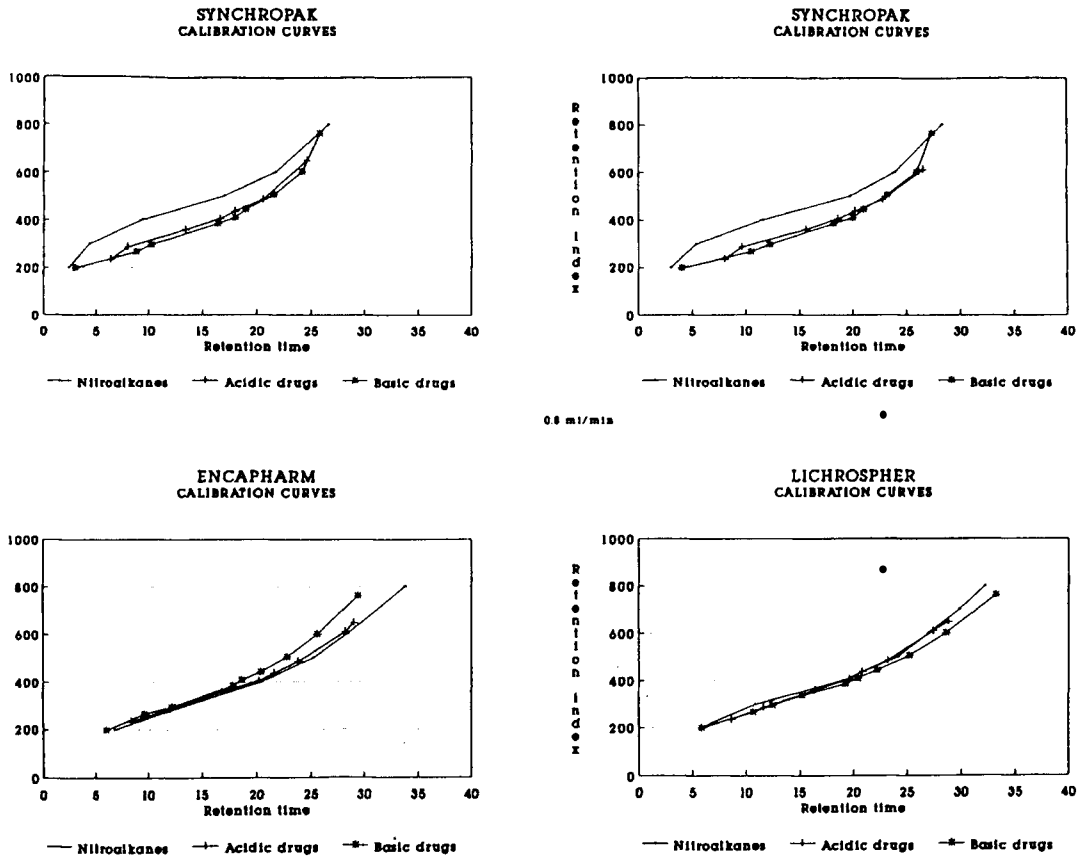

Fig.2. Calibration curves prepared for Synchropak SCD column, flow 0.8 ml/min and 1 ml/min, EncaPharm RP 18 and for Lichrospher RP 18 columns.

TABLE 2. RI_{NO2} VALUES OF DRUGS, CALCULATED IN THE 1-NITROALKANE SCALE.
LIB = LISTED VALUES ON SUPERSPHER RP-18 COLUMN FROM REF.36.

SUBSTANCE	LIB	SUPER	LICHRO	NUCLEO	INERT	ENCA	SELECT	SYNC-1	SYNC-2	Mean	SD
ATENOLOL	224	250		254	224	212	244	321	322	245	43,9
THEOBROMINE	229	254	249	256	232	218	258	366	363	253	57,2
AMPHETAMINE	242	256	249	262	241	232	248	252		244	9,8
CODEINE	247	267	254	270	241	230	266	350	351	261	46,4
ACETAZOLAMIDE	249	250	261	272	254	238	268	340		262	33,3
AMINOPHENAZONE	255	259	248	274	238	229	258	347	339	258	44,8
OXYCODONE	262	274		286	260	231	278		366	260	45,2
CAFFEINE	271	288	281	300	273	254	299	408	402	291	58,6
HYDROCHLOROTHIAZIDE	276	288	290	288	275	258	294	373	375	287	44,0
LIDOCAINE	286	286	299	304	277	267	294		351	280	27,1
STRYCHNINE	290	306	303	306	281	265	306	412	409	301	55,7
PHENAZONE	306	318	330	326	308	296	327	425	419	324	49,7
ACEBUTOLOL	309	324	312	324	308	291	322	428	422	322	52,8
SALICYLAMIDE	311	318	314	322	309	296	326	392	390	319	36,7
DIAMORPHINE	324	334	336	340	326	304	336	442	436	336	52,0
ASPIRIN	330	340	335	350	340	327	346	408	402	346	31,1
TILIDINE	336	346		347			346	452		373	52,8
DIBENZEPINE	350	357	363	356	341	326	356	459	451	356	50,1
PHENACETIN	357	364	372	362	358	348	368	456	452	369	43,2
PHENOBARBITAL	360	364	367	368	363	350	368	439	446	369	37,2
DROPERIDOL	372	378		371	359	344	376	482	474	379	56,1
PROPRANOLOL	372	378	386	374	362	344	366	466	460	372	45,6
CYCLOBARBITAL	372	381	376	378	377	366	378	462	456	384	38,7
FLURAZEPAM	386	388	394	383	375	361	384	489	478	388	48,6
MIANSERINE	390	394		386	375	363	390	491	483	395	52,5
CARBROMAL	393	398	396	400	398	390	396	476	472	405	36,1
NORMETHADONE	412	430	448	411	402	389	412	513	506	417	47,1
VINYLBITAL	413	410		416	417	401	412	492	491	421	39,5
PROMAZINE	415	422	433	405	399	384	408	509	499	412	46,6
PROPYPHENAZONE	416	421	443	425	429	406	434	515	502	430	39,6
METHADON	437	453	477	429	421	402	440	544	529	437	51,2
ALPRAZOLAM	443	434	465	442	445	428	454	547	547	451	48,7
PERPHENAZINE	450	447	463	424	431	410	428	534	515	437	45,1
TOLBUTAMIDE	462	468	470	470	472	461	476	554	548	478	38,0
CLOMIPRAMINE	466	480	497	452	450	432	460	561	550	462	47,7
THIOPENTAL	475	468		482	483	471	482	548	539	486	33,0
FLUPHENAZINE	477	478	495	452	459	437	458	565	547	465	46,6
LORMETAZEPAM	483	478	482	480	489	474	490	569	560	491	38,6
TRIFLUOPERAZINE	488	498	537	470	475	459	478	590	562	486	48,4
TRIFLUOROPROMAZINE	505	496		466	466	446	480	579	567	483	52,2
DIAZEPAM	517	508	529	508	528	516	524	586	580	525	30,8
TETRAZEPAM	530	548		520	548	537	562	557	539	544	14,0
WARFARIN	540	532		530	542	525	546	601		546	28,0
FLUNARIZINE	581	592		530	510	502	583	680	669	557	72,5
DICLOFENAC	607	610	610	610	614	590	630	694	690	616	39,2
IBUPROFEN	613	630	625	630	634	622	622	682	678	633	24,8
PHENYLBUTAZONE	640	654	662	660	655	649	670	734	734	665	35,6

MSD = 44,4

TABLE 3. RI^{D}_{NO2} VALUES OF DRUGS CALCULATED WITH REFERENCE DRUG MARKERS.
LIB =LISTED VALUES ON SUPERSPHER RP-18 COLUMN FROM REF.36.

SUBSTANCE	LIB	SUPER	LICHRO	NUCLEO	INERT	ENCA	SELECT	SYNC-1	SYN-0.8	Mean	SD
ATENOLOL	224	230		244	232	230	228	229	229	232	5,6
THEOBROMINE	229	234	236	236	232	230	228	253	253	234	9,8
AMPHETAMINE	242	236	243	249	250	251	232			246	8,0
CODEINE	247	247	247	254	251	249	246	244	246	249	3,2
ACETAZOLAMIDE	249	248	248	252	260	248	250	234		249	7,7
AMINOPHENAZONE	255	238	244	254	235	241	228	245	231	240	8,4
OXYCODONE	262	254		264	266	254	260		257	258	5,1
CAFFEINE	271	270	278	276	268	268	278	285	289	273	7,7
HYDROCHLOROTHIAZIDE	276	269	271	268	270	267	274	288	292	271	9,6
LIDOCAINE	286	268	283	280	281	283	276		247	279	13,0
STRYCHNINE	290	290	285	284	283	282	292	293	299	286	6,0
PHENAZONE	306	302	311	314	301	303	312	306	332	306	10,1
ACEBUTOLOL	309	310	294	308	306	303	308	312	313	307	6,1
SALICYLAMIDE	311	302	303	308	302	303	310	300	304	304	3,3
DIAMORPHINE	324	324	324	332	324	327	326	328	330	327	3,0
ASPIRIN	330	332	331	346	335	338	340	314	314	335	11,6
TILIDINE	336	336		341			340	351		342	6,4
DIBENZEPINE	350	345	350	350	345	347	348	347	350	347	2,1
PHENACETIN	357	358	362	362	355	357	358	363	365	358	3,5
PHENOBARBITAL	360	358	357	366	360	359	358	356	359	359	3,0
DROPERIDOL	372	374		373	367	368	370	374	377	370	3,6
PROPRANOLOL	372	374	361	372	370	368	358	355	361	367	7,0
CYCLOBARBITAL	372	377	372	378	374	374	370	369	371	374	3,2
FLURAZEPAM	386	384	390	383	372	370	376	398	397	378	10,7
MIANSERINE	390	384		388	387	390	384	384	388	387	2,4
CARBROMAL	393	396	389	400	395	397	394	385	389	395	5,0
NORMETHADONE	412	428	414	427	427	424	412	403	418	421	9,0
VINYLBITAL	413	407		414	410	409	409	403	408	409	3,3
PROMAZINE	415	420	410	420	423	416	408	401	406	415	7,9
PROPYPHENAZONE	416	418	443	423	423	414	434	431	431	421	9,4
METHADON	437	448	456	450	445	441	446	444	450	445	4,6
ALPRAZOLAM	443	432	459	442	440	437	452	469	460	443	13,0
PERPHENAZINE	450	442	453	443	458	451	430	426	432	444	11,7
TOLBUTAMIDE	462	457	463	474	468	472	476	466	470	470	6,2
CLOMIPRAMINE	466	476	474	478	480	477	474	464	475	475	4,8
THIOPENTAL	475	466		490	479	483	482	462	463	479	11,2
FLUPHENAZINE	477	476	473	480	492	483	468	472	466	480	8,5
LORMETAZEPAM	483	466	479	486	485	487	490	480	480	484	7,4
TRIFLUOPERAZINE	488	498	493	500	516	510	490	488	484	503	11,0
TRIFLUOROPROMAZINE	505	496		496	496	493	496	486	499	494	4,2
DIAZEPAM	517	508	496	524	526	535	524	505	506	524	13,5
TETRAZEPAM	530	548		530	548	557	558	467	463	540	41,6
WARFARIN	540	532		546	541	543	546	522		540	9,5
FLUNARIZINE	581	596		585	581	579	583	582	595	583	6,8
DICLOFENAC	607	608	608	620	613	603	614	598	605	608	6,9
IBUPROFEN	613	630	614	640	627	636	610	584	597	625	19,7
PHENYLBUTAZONE	640	654	652	670	655	660	644	623	636	653	14,6

MSD = 10,3

TABLE 4. THE DEVIATIONS FROM THE LIBRARY RI-VALUES, DETERMINED WITH 1-NITROALKANES AND DRUG STANDARDS. MEAN ± SD FOR 47 DRUGS EXAMINED ON 7 COLUMNS

	COLUMNS							
	SUPER	LICHRO	NUCLEO	INERT	ENCA	SELECT	SYNC-1	SYNC-2
RI_{NO2} mean dev.	-7.4	-14.2	-4.3	3.7	-8.0	18.5	-92.6	-87.6
$SD\text{-}NO_2$	8.8	11.6	17.3	15.4	16.3	12.6	23.2	21.4
RI^D_{NO2} mean dev.	0.5	-0.7	-5.3	-2.6	-1.8	-0.5	4.3	1.5
SD^D_{NO2}	8.7	9.0	8.2	8.9	9.5	9.2	15.1	16.5

cies, but similar precisions were noted in the case of octadecyl-silica columns from different manufacturers (Nucleosil AB, Inertsil and EncaPharm). The largest deviations from the listed values were observed for the Synchropak column, which was of different silica, different stationary phase and different length than the reference column. Thus, if the Synchropak column is disregarded, the standard deviations of the mean deviation in RI^D_{NO2} for all other columns were in the range of 8-10 RI units, indicating the precision available with this standardization procedure on an interlaboratory level. This would mean that data bases may be searched with a search window of $\pm$ 30 RI units (i.e. $\pm$ 3 times the standard deviation).

The results obtained with two different flow rates (0.8 and 1 ml/min) for Synchropak column showed only small differences. It may be assumed therefore, that small fluctuations in the flow rate do not distinctly contribute to the variability of results using the drug scale or the 1-nitroalkane scale.

CONCLUSIONS:

1.) The application of selected drugs as retention index markers gave comparable results in the determination of RI^D_{NO2} for acidic, neutral and basic drugs analyzed between two laboratories on various kinds of reversed-phase columns.

2.) Therefore, it is possible to use, exchange, and expand HPLC retention index data at the interlaboratory level when RI^D_{NO2} are determined in the above way.

3) The intra- and interlaboratory variability of RI values, obtained with different reversed-phase columns, had a standard deviation of ± 10 RI units.

4.) It is interesting that RI values determined in the above way on the octyl-silica column (Select B) did not significantly differ from those obtained on the octadecyl-silica columns (Superspher and Lichrospher) from the same manufacturer.

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