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Impact of urine matrix and isolation procedure on retention behaviour of basic drugs in thin-layer chromatography

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Abstract: The influence of three different isolation procedures, namely liquid–liquid extraction, Extrelut column extraction and XAD-2 column extraction, and of the urine matrix on the standardised Rf values and variability of Rf values of some selected basic drugs was investigated. It appears that the liquid–liquid extraction may give a significant deviation of standardised Rf values in respect of pure drugs, which is dependent on the TLC system. For all three isolation procedures, the search window for substance identification by means of data collection based on standardised Rf values of pure drugs should be slightly wider after extraction than when using pure drugs. The TLC system cyclohexane–toluene–diethylamine (75:15:10, v/v/v) showed the best accuracy and precision of Rf values.

Keywords: *Thin-layer chromatography; urine; screening for basic drugs; systematic toxicological analysis; variability of Rf values; extraction.*

Introduction

For the identification of substances, combinations of TLC systems, eventually in association with gas chromatography, are widely used. Because Rf values, which are used as identification parameters, may vary considerably from day to day and from laboratory to laboratory, standardisation of these Rf values is necessary. It has been shown that standardisation by means of 2, 3 or 4 reference substances, which are carefully chosen for each system and which are run on each plate, give an enormous improvement in variability [1–3]. By this means of standardisation, Rf values are transformed into corrected Rf values (Rf^c values). As these Rf^c values appeared to be rather stable, it became useful to collect data for a great many substances with the purpose of interlaboratory use [2, 3]. Although the Rf^c values in these data bases are obtained in a standardised way on the interlaboratory scale, there remains a certain variability. When these data bases are used for identification purposes, one has to determine a "search window" which has to be obtained on the basis of a reliable estimation of the interlaboratory variability of the Rf^c values. The latter, which are usually determined using pure drugs, are given together with the data bases [2, 3].

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It has been realised that in biomedical and clinical analyses, the drug is not pure but present in a biological matrix [4]. Endogenous components from the biological matrix, isolated together with the drug of interest, are also spotted on the plate and may influence retention of the drug.

Recently, the influence of blood and liver, as examples of forensic toxicological matrices, on interlaboratory variability of Rf values was investigated [4].

The purpose of the present study was to examine in a similar way for biomedical and clinical analyses the impact of the urine matrix on the retention behaviour of substances. A number of basic drugs were selected, together with five different TLC systems, for which data bases were available. In addition three isolation procedures were chosen on basis of their differences in methodology and usefulness: a liquid–liquid batch extraction, a column extraction based on partition and a column extraction based on absorption.

Experimental

Methods

Samples of urine of a volunteer were individually spiked with fluphenazine, imipramine, levomepromazine, lignocaine (lidocaine), nitrazepam and nortriptyline to a final concentration of 5 mg l^{-1} of each drug. The drugs used were pharmaceutical grade and used as received.

The samples were then extracted by one of three methods:

Liquid–liquid extraction. Five millilitres of urine adjusted to pH 3 with tartaric acid, was extracted twice with 10 ml of ether. The organic phase was discarded and the remaining water phase was made alkaline with ammonia and was extracted with 10 ml of dichloromethane–isopropanol (4:1, v/v). The organic phase was dried with sodium sulphate.

Extrelut extraction. Five millilitres of urine, mixed with 5 ml of 5% (m/v) ammonia, was put on the Extrelut column. After 15 min the column was eluted with 25 ml of dichloromethane.

XAD-2 Extraction. The XAD-2 column was washed with acetone, then with water and finally with ammonia. Five millilitres of urine mixed with 5 ml of 5% (m/v) ammonia was put on the column followed by 4 ml of 5% (m/v) ammonia. The aqueous eluate was discarded and the column dried with air. The drugs were eluted with 15 ml of ethyl acetate. The eluate was passed through a phase-separation filter (1PS, Whatman).

The eluates of these extractions were evaporated under a gentle stream of nitrogen at 40°C; the residues of these extractions were reconstituted in 150 µl of ethyl acetate and 10-µl aliquots were spotted on silica gel plates together with aliquots of pure drug standards and developed over a distance of 8 cm in one of five TLC systems:

1. Methanol, unsaturated chamber, according to Machata [5];
2. Methanol–butanol (60:40, v/v) with 0.1 M sodium bromide, unsaturated chamber, an ion-pair system, according to De Zeeuw *et al.* [2].
3. Chloroform–methanol (90:10, v/v), saturated chamber, plates impregnated with KOH, as recommended by Stead *et al.* [3];

4. Cyclohexane–toluene–diethylamine (75:15:10, v/v/v), saturated chamber, plates impregnated with KOH, as recommended by Stead and coworkers [3];
5. Ethyl acetate–methanol–ammonia 25% (m/m) (85:10:5, v/v/v), saturated chamber, according to Davidow [6].

The chromatograms were inspected in UV light as well as after spraying with acidified iodoplatinate reagent. The Rf values of the spots were corrected using for each system a mixture of three reference substances which was applied on each plate [2]:
TLC-1: codein [20], flurazepam [52], papaverine [74];
TLC-2: codeine [22], diphenhydramine [48], diazepam [85];
TLC-3: desipramine [11], caffeine [58], meclozine [79];
TLC-4: hydroxyzine [9], pethidine [37], trimipramine [62];
TLC-5: morphine [20], quinine [42], haloperidol [74].

The values in parentheses are the hRf (100 × Rf) values of the substances in the particular system obtained from the data bases.

Extractions for each substance were performed in triplicate and each extract was spotted on three plates run on different days.

Materials

The reagents used were analytical grade and obtained from Merck (Darmstadt, FRG). The silica gel plates contained fluorescence indicator (GF254, Merck). The Extrelut columns were glass columns (200 × 20 mm i.d.) with a glass frit filled with 5 g of Extrelut material (Merck). The XAD columns were glass columns (300 × 10 mm i.d.) with stopcocks filled with a piece of glasswool and 5 g of XAD-2 material (particle size 0.3–1 mm, Serva, Heidelberg, FRG). The XAD-2 material was purified previously by Soxhlet extraction for at least 8 h with methanol.

Results and Discussion

Table 1 and Fig. 1 present the deviation of hRf^c values ($Rf^c \times 100$) found in this study for pure substances from their reference values in the data bases. Also, the day-to-day standard deviations observed in this study are given.

The reference values in the data bases were for the systems methanol and ethyl

Table 1
Reference hRf values from literature from five TLC systems compared with the mean values found for the pure drugs found in this study

Drug	TLC systems: methanol		methanol butanol bromide		chloroform methanol (KOH)		cyclohexane toluene DEA (KOH)		ethyl acetate methanol ammonia	
	R	F	R	F	R	F	R	F	R	F
nortriptyline	10	7.4	71	72.7	16	14.2	27	28.0	45	52.2
imipramine	21	18.1	47	40.1	23	35.0	49	49.7	68	70.7
levomepromazine	31	32.6	49	47.1	38	45.6	49	48.0	79	79.3
fluphenazine	44	46.9	49	43.3	23	32.3	6	5.2	44	45.3
lidocaine	68	72.4	69	71.0	73	70.7	35	31.7	80	76.0
nitrazepam	84	81.8	86	86.2	36	52.8	0	0.0	59	54.2

R, reference hRf value.
F, Mean hRf^c value for the pure drugs found in this study.

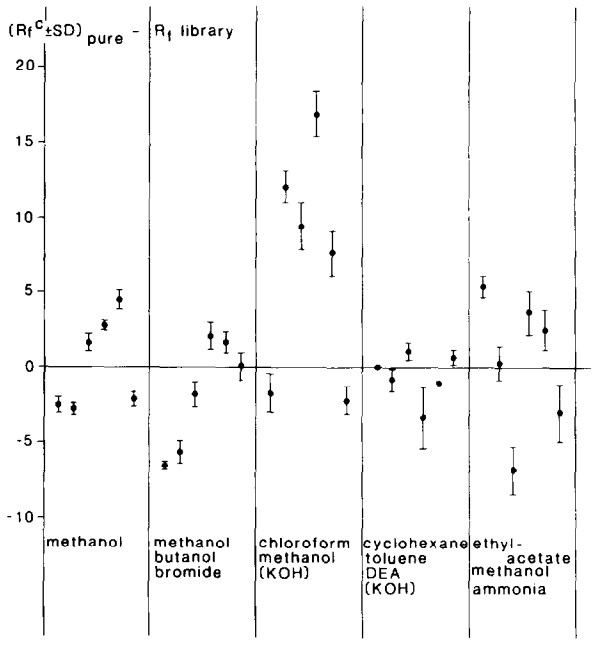

Figure 1
Deviation of hRf values found for pure drugs from their reference values in data bases together with observed day-to-day standard deviations (SD). The bars show ±1 SD. When no bars are present the SD = 0. For each TLC system the drugs are ordered from left to right according to increasing Rf values from the literature (see Table 1).

acetate–methanol–ammonia from an interlaboratory survey by the Senate Committee on Clinical Toxicological Analysis of the German Research Foundation and for the chloroform–methanol (KOH) systems and cyclohexane–toluene–DEA (KOH) from Stead and coworkers [3]. The reference values in the methanol–butanol–bromide system were determined in one laboratory [2].

In Fig. 1, for each system, the substances were arranged from left to right in ascending order, in order to see if changes are dependent on the Rf value itself, i.e. the position of the substance on the plate in relation to the possible presence of interfering endogenous components.

The accuracy (i.e. nearness to the data base value) is best for the systems cyclohexane–toluene–DEA and methanol with a largest deviation of less than 5 hRf units, followed by the systems methanol–butanol–bromide and ethyl acetate–methanol–ammonia with a deviation of less than 8 hRf units. The system chloroform–methanol has a largest deviation of over 19 hRf units.

Moreover, in the system chloroform–methanol it appeared that substances with Rf values in the middle part of the plate give increased Rf values in relation to the database values whereas Rf values substances with low or high Rf values decrease slightly. However, considering the low number of substances this effect may not be significant.

With regard to precision (i.e. scatter around the mean) the system methanol comes out best followed by the systems methanol–butanol–bromide and cyclohexane–toluene–DEA.

In an earlier study [4] on the influence of liver and blood matrices similar results were obtained for the TLC systems methanol, methanol–butanol–bromide and chloroform–methanol. The other two systems were not included in that study.

The effect of the isolation procedure on the accuracy of Rf values for the given TLC systems is shown in Fig. 2. Here, the Rf^c values of the drugs after extractions are compared with those of the pure drugs spotted on the same plate. In this way, variability caused by differences in temperature, humidity, etc. is eliminated. For clarity the standard deviations are not presented in this figure, as it appeared that the reproducibility for each system is in the same range as the pure drugs presented in Fig. 1.

Figure 2 shows that in the TLC system cyclohexane–toluene–DEA extraction has virtually no influence on Rf^c values, irrespective of the isolation method. In the other systems deviations from pure drugs were found to be larger and somewhat dependent on the different procedures.

In three systems: methanol, methanol–butanol–bromide and chloroform–methanol systems, liquid–liquid extraction gave the highest variability; with the systems methanol and methanol–butanol–bromide systems a significant decrease in Rf value was observed; with the chloroform–methanol system low Rf values were increased whereas higher Rf values were decreased.

Extrelut extractions and XAD-2 extractions gave better results than liquid–liquid extractions, with the exception of XAD-2 in the ethyl acetate–methanol–ammonia system. For all systems hRf values obtained after Extrelut extraction deviate less than 1.5 unit from those measured for pure drugs.

When comparing Figs 1 and 2, taking into account the difference in the scale of the ordinate, it is clear that the deviation of Rf values due to the isolation procedure and the

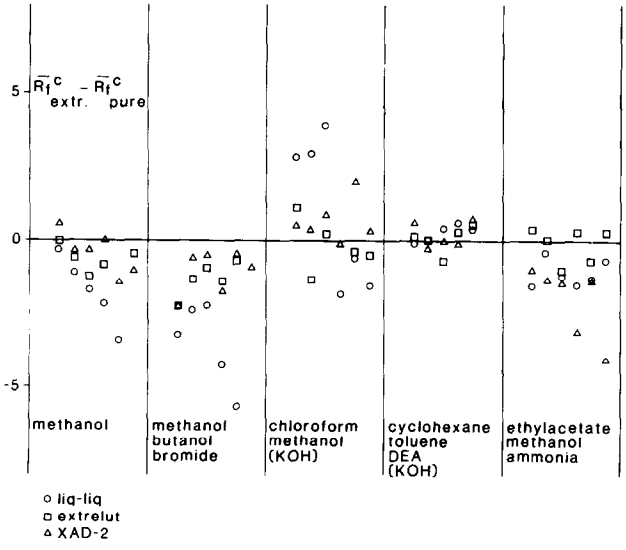

Figure 2
The influence of isolation procedure on hRf values compared with those of pure drugs spotted on the same plate. For each drug the difference between the mean standardised hRf value of the drug in pure solution and after extraction from urine using three isolation procedures are depicted: ○ liquid–liquid extraction, □ Extrelut extraction and Δ XAD-2 extraction. The drugs are in the same order as in Fig. 1.

urine matrix are small compared to the lack of accuracy of Rf values in the data base in relation to measured Rf values. Furthermore, the day-to-day and interlaboratory variability is larger than the variability caused by the urine matrix. Therefore, the search window for substance identification derived from the data base for pure drugs, should be slightly wider for drugs after extraction than for pure drugs. This is in agreement with earlier studies with blood and liver matrices.

In case of doubt, additional evidence may be achieved by co-spotting on the same plate the pure substance, found to be a candidate for identification, and the urine extract.

It should be noted that the individual TLC systems have a different effect on extracted endogenous urine components and on the spot shape of the drugs. The methanol system gave relatively diffuse spots, especially for substances with high Rf values, resulting in a low sensitivity. In contrast, the cyclohexane–toluene–DEA system gave small, concentrated spots for all drugs tested.

Because of its high accuracy of Rf values after extraction and good accuracy and precision of the Rf values for pure drugs compared with the data base values, the cyclohexane–toluene–DEA system appears to be the best. However, the need of plate impregnation with KOH prior to development is a drawback. The latter also applies to the chloroform–methanol system, which showed the lowest accuracy and precision in this investigation. The other three systems do not need prior impregnation and provide acceptable accuracy and precision for pure drugs as well as for drugs after isolation from urine.

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