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ON-LINE COMBINATION OF DIALYSIS AND COLUMN-SWITCHING LIQUID CHROMATOGRAPHY AS A FULLY AUTOMATED SAMPLE PREPARATION TECHNIQUE FOR BIOLOGICAL SAMPLES

DETERMINATION OF NITROFURAN RESIDUES IN EDIBLE PRODUCTS

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SUMMARY

The potential of dialysis coupled on-line with trace enrichment by column-switching high-performance liquid chromatography as an automated sample treatment technique in drug residue analysis has been investigated. The nitrofuran veterinary drugs furazolidone, nitrofurazone, nitrofurantoin and furaltadone were used as model compounds. Critical parameters, *i.e.*, dialyser dimensions, air segmentation, dialysis time, flow-rates and enrichment column breakthrough, were evaluated. Depending on the analytical purpose, the technique can be set up in either a highly sensitive or a high-speed mode. High dialysis efficiencies (> 85%) can be obtained in a stopped-flow dialysis of only 3 min. Aqueous biological sample volumes (eggs, meat, milk) of 100 μl to 4 ml can be injected with only minor sample treatment. A routine monitoring method for residues of nitrofuran drugs in edible products was set up. A 4-ml sample was dialysed in a pulsed mode with an efficiency of about 30% and concentrated on a short C_{18} column. Recoveries compared with standards were 75–85% (coefficient of variation 2–7%). Limits of determination ranged from 1 to 10 μg/kg. At this concentration level, *ca.* 30 samples can be monitored per day.

INTRODUCTION

In drug residue analysis, sample pretreatment is an essential part of the analytical procedure aimed at (i) the removal of substances that interfere with the detection of the analyte or deteriorate the chromatographic columns used and (ii) the enrichment of the analyte. Most drug residue methods described in the literature use a combination of some form of deproteination, liquid–liquid extraction and/or off-line

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solid-phase extraction, before the biological sample is subjected to a chromatographic separation. Although these procedures can all, in principle, be automated[1,2], in actual practice many, mainly technical, problems arise because of the complex heterogeneous matrix. For the relatively simple urine and plasma matrix, repeated direct injection of a limited volume of the crude sample onto a column-switching reversed-phase high-performance liquid chromatographic (HPLC) system is possible (*e.g.*, refs. 3 and 4). When short, highly selective affinity chromatographic columns are used containing, *e.g.*, immobilized antibodies, even large volumes (>20 ml) can be injected[5]. This approach does not work for more complex samples. In our laboratories, the combination of two short precolumns, one containing a C_{18}-bonded phase and the other a polymer-based material, was studied for the determination of nitrofurans in meat and eggs. Despite aqueous deproteination, via salting out or mineral acid addition, and dilution, the precolumns were more or less coated with the sample matrix, which resulted in clogging and a rapid loss of efficiency. Another, not yet fully explored, approach to on-line sample purification, size exclusion[1], appears to have a number of drawbacks, such as the large column volume required for sample volumes exceeding 1 ml[6], the occurrence of stationary phase–analyte interactions other than size exclusion and the often relatively low pressure resistance.

As an alternative, dialysis can be used for the on-line removal of proteins and other macromolecular compounds. Although diffusion is the major driving force and equilibrium, under static conditions, is reached only after many hours, dialysers were introduced in continuous-flow (CF) analyser systems about 15 years ago[7]. The low, but very reproducible, dialysis efficiencies obtained (1–10%) limited the applicability to samples with a relatively high analyte concentration[8] or analytes that have favourable detectability characteristics[9]. These limitations were overcome by trace enrichment of the CF dialysate using column-switching techniques[10–15]. Recently, a modification of CF dialysis that uses an on-line stopped-flow dialysis of small plasma samples was reported[16]. This improved the dialysis efficiency to 30–50%, while maintaining a short analysis time. This study was initiated to investigate the potential of different modes of on-line dialysis coupled with column-switching HPLC for the fully automated purification and enrichment of complex biological samples containing

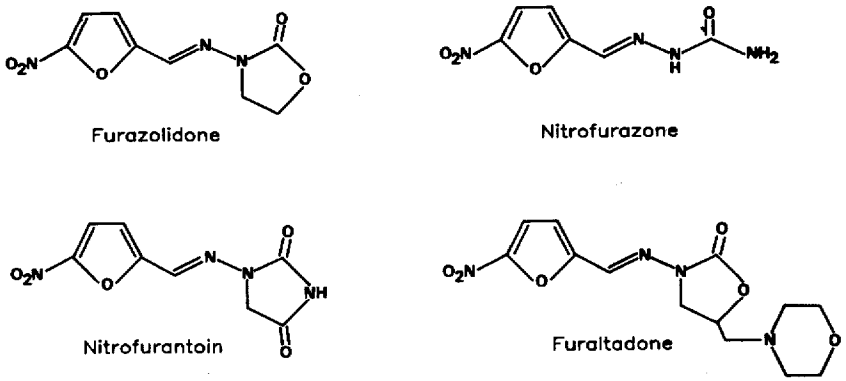

Fig. 1. Structures of the nitrofuran drugs furazolidone (FU), nitrofurazone (NZ), nitrofurantoin (NT) and furaltadone (FT).

very low analyte concentrations. As an illustration, a routine monitoring method was developed for residues of nitrofuran drugs (Fig. 1) in edible products.

In the literature, a number of gas chromatographic[17], thin-layer chromatographic[18] and HPLC[19–27] methods have been described for the low-level (5–50 μg/kg) determination of residues of one or more of these suspected mutagenic[28–30] compounds in biological samples. Although the stated limits of detection are, in some instances, adequate for monitoring an individual drug in an individual sample matrix[18,27], none of the methods can be used for routine multi-residue screening.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Standards of furazolidone, nitrofurantoin, nitrofurazone and furaltadone were obtained from Sigma (St. Louis, MO, U.S.A.). The HPLC eluent was prepared by mixing 800 ml of a 0.1 *M* sodium acetate–acetic acid buffer (pH 5) with 200 ml of acetonitrile. Water was purified with a Milli-Q purification system (Millipore, Milford, MA, U.S.A.). Standard stock solutions contained 100 μg/ml in acetone–methanol (1:1); standard working solutions were prepared from a 10 μg/ml saline solution. Water was used as the acceptor phase in the dialysis; it was also used to flush the donor and acceptor channels.

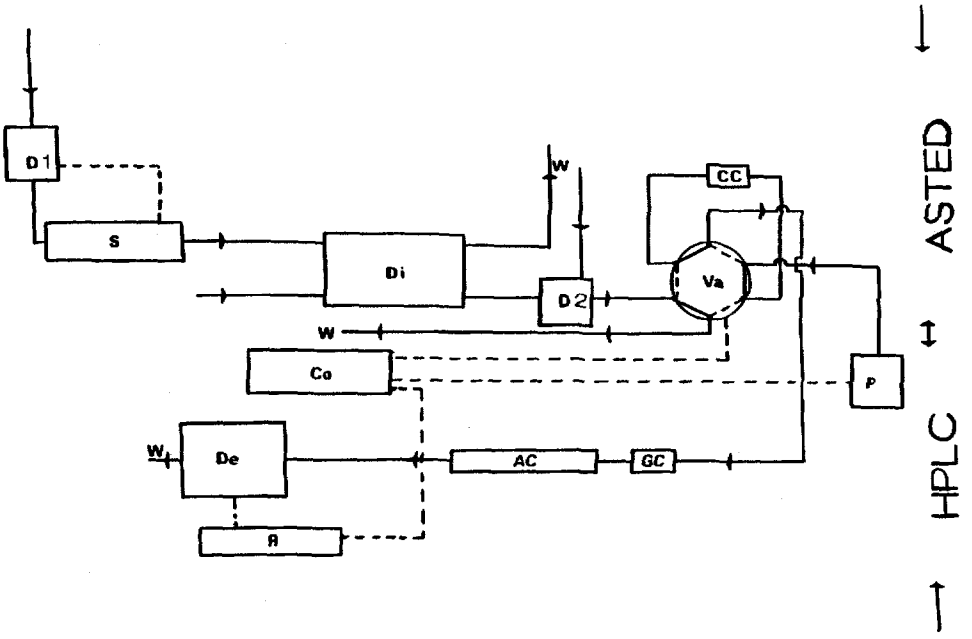

Fig. 2. Schematic representation of the on-line dialysis–column switching HPLC system used. The sample is introduced with dilutor D_1 and sampler S to the dialysis module Di. The dialysis acceptor solvent is transported with diluter D_2 to the enrichment column, CC, through the six-port valve Va. The enriched sample is backflushed with HPLC eluent to the analytical column AC (with guard column, GC) with pump P. After the HPLC separation, the analytes are detected by the UV–VIS detector, De, and recorded by a recorder or intergrator, R. All timed events are handled by the ASTED controller (Co).

Instrumentation and chromatographic conditions

The instrumentation consisted of an autosampler–dialysis module and a column-switching HPLC module. The general configuration is depicted in Fig. 2. In all experiments, the column-switching HPLC system consisted of a liquid chromatographic pump capable of maintaining a constant pulseless flow of 0.2–2 ml/min (Model 302/5 SC; Gilson Medical Electronics, Villiers-le-Bel, France), a UV–VIS absorbance detector (Spectroflow 783; ABI-Kratos, Ramsey, NY, U.S.A.) and an automatic six-port valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.) mounted either on a switching-valve module (Must; Spark, Emmen, The Netherlands) or on a autosampling injector (Model 231; Gilson). Chromatographic peaks were registered with a recording integrator (Chromatopac C-R2A; Shimadzu, Kyoto, Japan). All timed events were controlled with a Model 450 (ABI-Kratos) system controller. The analytical column was a 250 × 4.6 mm I.D. column containing 5-μm Hypersil ODS (Chrompack, Middelburg, The Netherlands). The precolumn used in the final procedure was a 60 × 4.6 mm I.D. column fitted with 20-μm frits and containing 37–50-μm Bondapak C_{18}/Corasil (Millipore). In the optimization experiments, 50–100-μm polymer XAD-4 material was also used for trace enrichment (Serva, Heidelberg, F.R.G.). The HPLC eluent flow-rate was 1.0 ml/min and the detector setting was 0.001 a.u.f.s. at 365 nm.

The autosampler–dialysis module was a modified Gilson ASTED system consisting of a Model 231 autosampling injector, two Model 401 dilutors equipped with 5- and 1-ml syringes and poly(methyl methacrylate) flat-plate dialyser blocks fitted with a Cuprophan membrane (10 000–15 000 dalton cut-off) having donor volumes of 100 and 370 μl and acceptor volumes of 175 and 650 μl, respectively (Type I).

For the experiments dealing with dialyser dimensions and air segmentation, a continuous-flow system was used consisting of a Model SA 1000 automatic sampler (Skalar, Breda, The Netherlands), a Model 2002 peristaltic tubing pump (Skalar) and 24-in. flat-plate dialyser blocks fitted with a Type C cellulose acetate membrane, having donor and acceptor volumes of 750 μl (Technicon, New York, NY, U.S.A.) (Type II).

For the extraction of biological samples, a Model 400 Stomacher laboratory blender (Lameris, The Netherlands) was used.

Set-up of the on-line dialysis column-switching HPLC system and modes of dialysis

On-line dialysis was performed according to different modes. In the continuous-flow (CF) mode, the entire sample is transported across the dialysis membrane through the donor channel at a fixed continuous flow-rate. Hence the dialysis takes place while both the sample and the acceptor solvent are moving. In this mode, air segments are usually introduced into the sample stream in order to improve mass transfer. In the static mode, the donor channel is filled with the sample but now the flow is stopped and the sample is statically dialysed against a continuously moving acceptor flow for a certain period of time. In the pulsed mode, the sample has a volume exceeding the donor channel volume and is divided into several aliquots (pulses) which are successively analysed in the static mode.

The Gilson ASTED cycle consists of four phases. First, an aliquot of the sample solution is transported from the sample cup to the dialyser donor channel with a 5-ml syringe diluter. A maximum of about 4 ml of sample can be introduced either as a

single continuously flowing injection or divided into multiple, pulsed injections. Each sample plug is bracketed with small air plugs. During the dialysis of the continuous-flowing or static sample, the dialyser acceptor stream is continuously transported with a second, 1-ml syringe, diluter and successive 1-ml volumes of the dialysate are led to the trace-enrichment column. After application of the total dialysate, the enrichment column can be flushed with acceptor stream solvent and, after switching of the automatic six-port valve, backflushed to the analytical column by eluting with the HPLC eluent. The dialyser donor and acceptor channels are then flushed, the six-port valve is returned to its original position and the enrichment column is regenerated with acceptor-stream solvent.

Sample preparation

Caution. Because of the sensitivity of nitrofurans to light, one should conduct all the steps in the analytical procedure, including sampling and dialysis, in the absence of daylight or artificial white light.

Milk. A raw milk sample is decreamed by centrifugation (10 min, 2000 *g*) and subsequent freezing (15 min, −20°C). An aliquot (10 ml) is diluted with 10 ml of saline solution (0.9% sodium chloride in water) and 2 ml of an aqueous 1% sodium azide solution.

Meat. A 10-g amount of homogenized veal or chicken meat is blended (3 min) in a Stomacher laboratory blender with 30 ml of saline solution. After centrifugation (2000 *g*), 20 ml of the clear upper phase are isolated and mixed with 2 ml of 1% sodium azide solution.

Eggs. A 10-g amount of homogenized whole egg is diluted with 10 ml of saline solution and 3 ml of an aqueous 10% sodium azide solution.

The centrifuged and/or diluted solutions can be injected directly into the dialysis module.

Quantification

With each series of biological samples, a number of spiked samples was analysed. These were prepared by injecting a small volume (1–100 μl) of an aqueous standard solution into blank material. The drug concentrations were determined by comparison of peak areas or peak heights.

RESULTS AND DISCUSSION

Chromatography

The conditions used for the HPLC separation were a modification of the conditions described by Petz[19]. The sodium acetate buffer concentration was increased to 0.1 *M* in order to improve both the separation of the four drugs and the peak shape of furaltadone. With this eluent system a good separation was obtained on various HPLC columns, *i.e.*, 5-μm Hypersil ODS (250 × 4.6 mm I.D., Chrompack), 10-μm Cp-Spher C_{18} (250 × 4.6 mm I.D., Chrompack), 5-μm Supelcosil LC-8 or 5-μm LC-8 DB (150 × 4.6 mm I.D., Supelco, Bellefonte, PA, U.S.A.). The Hypersil ODS column was used in the optimization experiments. In Fig. 3 a typical chromatogram is shown, which was obtained after dialysis and subsequent enrichment and backflushing to the analytical column of an aqueous standard solution.

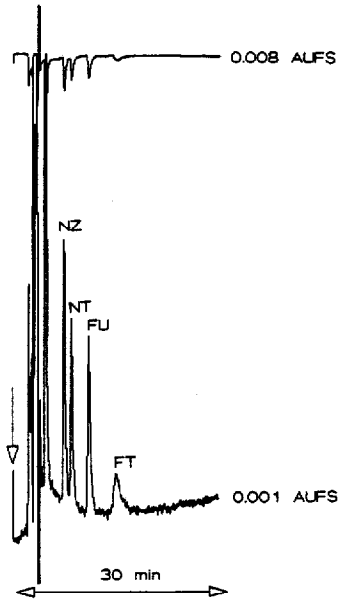

Fig. 3. HPLC trace obtained after injection of 500 μl of an aqueous standard solution, containing 0.005 μg/ml each of the four nitrofurans, to the on-line dialysis–column switching HPLC system. A Type I 370-μl dialyser block was used with an acceptor flow-rate of 0.36 ml/min and a static 3-min dialysis time. Other conditions are described under Experimental.

Factors influencing the system performance

A number of factors will influence the overall performance of the dialysis–HPLC system. Because the dialysis is mainly governed by molecular diffusion, the efficiency will depend on the concentration gradient across the membrane, the viscosity of the solution, the molecular weight of the analyte, the dialysis time, the dialysis contact surface per sample volume and the free fraction of analyte available for diffusion across the membrane. This can also be seen from Fick's first law of mass action:

$$dS/dt = -AD\frac{dC}{dx} \quad (1)$$

where dS/dt is the mass transfer per unit time, dC/dx is concentration gradient across the membrane, A is the dialysis contact surface and D is the diffusion coefficient of the analyte.

Because, inevitably, the dialysis step results in dilution of the analyte, the enrichment characteristics of the short precolumn coupled on-line with the dialysis unit should also be considered. In all the optimization experiments except those on the investigation of the dialysis time, Bondapak C_{18} material was used in the precolumn. The following parameters will be discussed: dialyser design, donor flow-rate, acceptor flow-rate and pulsed dialysis.

Dialyser design

The main features of dialyser blocks that influence the dialysis efficiency are the

TABLE I

DIALYSER-BLOCK DESIGN RELATED TO DIALYSIS EFFICIENCY IN THE CONTINUOUS-FLOW MODE

Parameter	*Type I*	*Type II*
Length (mm)	863	610
Width (mm)	2.0	1.7
Donor channel depth (mm)	0.2[a]	0.75
Acceptor channel depth (mm)	0.75[a]	0.75
Donor channel volume (μl)	470[a]	750
Acceptor channel volume (μl)	825[a]	750
Contact surface area (mm^2)	1726	1037
Contact surface area ($mm^2/\mu l$)[b]	3.7	1.4
Relative furazolidone response (A.U./μl)[c]	3.7	1.0
Relative dialysis efficiency (A.U./mm^2)[d]	100	60

[a] According to the producer's specifications.

[b] Membrane contact surface per unit donor channel volume.

[c] Relative furazolidone UV response (365 nm) obtained per unit donor channel volume filled with furazolidone standard solution (50 μg/ml, $n = 5$).

[d] Relative furazolidone response obtained per mm^2 of membrane contact surface area.

dialysis contact surface per sample volume and the ratio of donor and acceptor volumes. Two types of commercially available dialyser blocks were tested, using fully comparable membranes. These blocks were placed in a classical continuous-flow system[15] using air segmentation, and 6-ml aliquots of an aqueous standard solution of furazolidone were led through them, dialysed at donor and acceptor flow-rates each of 0.6 ml/min, enriched on the precolumn, separated on the HPLC column and detected.

Table I shows the characteristics of the dialyser blocks used and the results obtained in the experiments. The asymmetrically designed Type I block with a relatively wide but shallow donor channel has a 2.6-fold higher dialysis contact area per unit volume than the classical symmetrical Type II block. With this increased contact surface area, a 3.7-fold higher dialysis efficiency per 1-ml donor channel volume is obtained. It can then be calculated that the Type I dialyser block gives a 40% higher dialysis efficiency per 1 mm^2 contact area, probably as a result of better mass transfer in the donor phase because of the high linear velocity and the higher acceptor-to-donor channel volume ratio. All these experiments were carried out in a CF dialysis mode where the mass transfer in the donor phase is facilitated by the applied flow. For static dialysis, the gain in efficiency using the Type I dialyser blocks will probably be even higher. Unfortunately, because of technical limitations, we were not able to compare the two types of blocks in this dialysis mode using the ASTED module.

Donor flow-rate

Continuous flow dialysis. Traditionally, in CF analysers both the donor and acceptor flow move in a continuous mode, either concurrent or countercurrent. The flow-rate of the donor phase will determine the dialysis contact time per unit sample volume. In addition, the mass transfer will be influenced. Fig. 4 shows the responses obtained for the four nitrofurans when 4 ml of an aqueous standard solution were

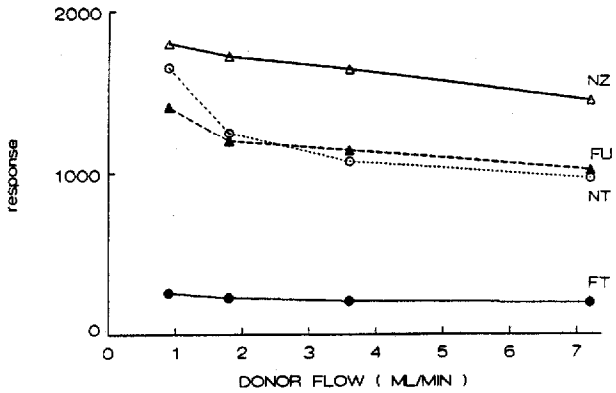

Fig. 4. Plot of the relative UV response (365 nm) obtained when 4-ml aliquots of an aqueous nitrofuran standard solution were applied in the continuous-flow mode to the on-line dialysis–column switching system at increasing donor flow-rates. The acceptor flow-rate was 0.36 ml/min in all instances.

transported with increasing donor flow-rates, at a constant acceptor flow-rate of 0.36 ml/min. Under the experimental conditions used, the lowest donor flow-rate that could technically be achieved was 0.9 ml/min. Under the given conditions laminar flow profiles are obtained. The collected dialysate was enriched on the short precolumn and backflushed to the analytical column. No air segments were introduced into the sample stream because of technical limitations.

In the donor flow-rate range investigated, the response is almost constant, indicating that the effect of the decreased dialysis contact time is balanced by an improved mass transport from the donor phase to the membrane. When the same sample volume was dialysed under static donor flow conditions by letting eight successive 500-μl pulses be dialysed for 3 min, a 2–3-fold higher response was obtained. It is therefore likely that for continuous donor flow-rates below 0.9 ml/min an increased efficiency will be observed, as is also indicated by the data points at the lower limit of donor flow in Fig. 4.

The dialysis efficiency is 10–15% for the donor flow-rate range now investigated. This efficiency is defined as 100 times the ratio of the response obtained from a sample containing a specific amount of analyte after quantitative collection of the dialysed fraction by the precolumn, and the response obtained after direct injection onto the precolumn of a small volume containing the same amount of analyte under conditions where no breakthrough occurs.

Air segmentation. In classical CF systems, air segmentation of solvent streams is often used to improve mixing of reagents, to separate successive samples, to diminish sample dispersion and/or to clean the tubing[7]. In previous studies on the CF dialysis[14,15] of biological samples containing veterinary drug residues, we also applied air segmentation. The ASTED system used in this study can only bracket the entire sample plug with air bubbles, mainly to diminish dispersion during static dialysis. In order to investigate the effect of air segmentation on dialysis efficiency under CF conditions, two series of experiments were performed with a classical CF system using Type II dialyser blocks. Replicate (n = 5) 6-ml samples containing various concentrations (10–50 μg/l) of the nitrofuran drugs were analysed and further processed, both

with air segmentation (5 bubbles/min) and without. One sample series consisted of aqueous standard solutions and a second series consisted of spiked meat sample extracts.

For the standard solutions, an average gain in efficiency of only about 5% was obtained when using air segmentation, whereas for the biological samples the efficiency under non-air-segmented conditions was only half of that obtained with introduction of air segments. This remarkable difference may be caused by the high concentration of solutes that can be retained at the membrane surface in the crude meat sample extracts. The so-called gel layer that is formed will negatively influence the mass transport across the membrane. These solutes (lipids, proteins) are apparently effectively removed from the surface by the bolus flow (longitudinal mixing) introduced by the air plugs. With the standard solutions, the formation of a gel layer will be only marginal. One may therefore conclude that when more or less crude, viscous samples are dialysed in a CF mode, air segmentation will be beneficial. Fouling of membrane and tubing can be prevented by periodically (every five samples) flushing the system with a 10% acetic acid solution. Additionally, the system should be flushed daily with acetonitrile–water (1:1).

Static dialysis. In order to investigate the influence of the dialysis time, a series of experiments were performed under static conditions with increasing donor channel residence times (Fig. 5). Because of the relatively large (up to 7.6 ml) volume of the acceptor phase led to the enrichment column, this column was filled with 50–100-μm XAD-4 polymer material in order to prevent breakthrough of the analytes. The analyte responses are seen to increase slowly with increasing residence time and to become constant after about 15 min. This can be explained by the rapidly decreasing analyte concentration in the donor phase which results in a diminishing return per unit time. In practice, a static dialysis time between 3 and 9 min will be a good compromise. In that case, dialysis efficiencies of between 30 and 50% are achieved at an acceptor flow-rate of 0.36 ml/min.

Acceptor flow-rate

The acceptor flow-rate directly influences the concentration gradient across the membrane, because it governs the volume against which the sample is dialysed. A

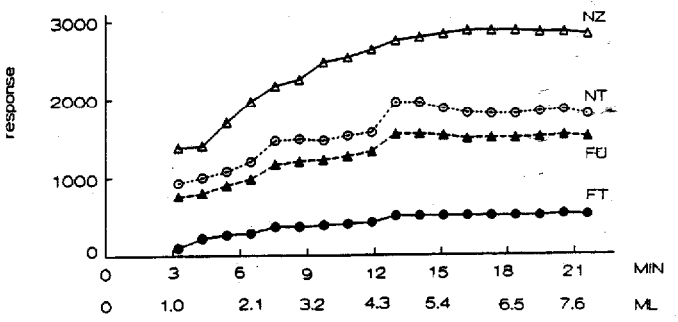

Fig. 5. Plot of the relative UV response (365 nm) obtained when 500-μl aliquots of an aqueous nitrofuran standard solution were dialysed in the static mode at increasing dialysis times. In all instances the acceptor flow-rate was 0.36 ml/min. The dialysis time and the total acceptor solvent volumes thus applied to the enrichment column are indicated on the abscissa.

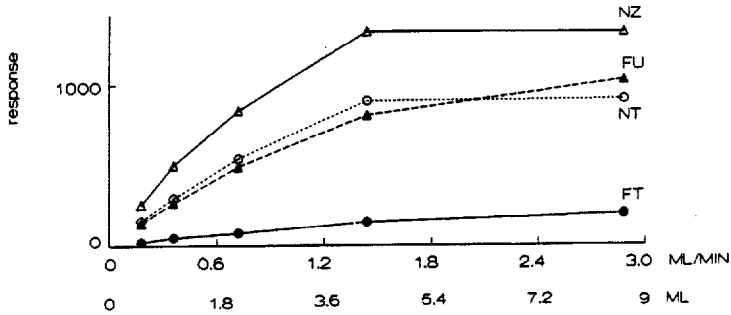

Fig. 6. Plot of the relative UV response (365 nm) obtained when 470-μl aliquots of an aqueous nitrofuran solution were dialysed in the static mode at increasing acceptor flow-rates. In all instances the dialysis time was 3 min. The acceptor flow-rate and the total acceptor solvent volumes thus applied to the enrichment column are indicated on the abscissa.

high acceptor flow-rate will result in rapid removal of the dialysed analytes, thereby establishing a permanent non-steady state but, unfortunately, also in a relatively large dilution. The results in Fig. 6 show that a high acceptor flow-rate indeed results in an increased dialysis efficiency. At an acceptor flow-rate of 0.18 ml/min, the acceptor channel is flushed with only 0.54 ml of water during the 3-min static dialysis of the 470 μl of sample solution. This increases to 8.7 ml at an acceptor flow-rate of 2.9 ml/min.

The almost linear increase in response stops at a flow-rate of about 1.5 ml/min. This can be explained by calculating the dialysis efficiencies. This was done according to the procedure described under *Donor flow-rate*. The average dialysis efficiencies obtained were 20% (0.18 ml/min), 30% (0.36 ml/min), 52% (0.72 ml/min) and 85% (1.44 ml/min). The last, high, dialysis yield shows that on-line dialysis is not only a very efficient means of removing macromolecular compounds within a few minutes, but is also capable of doing this without excessive loss of dialysable analytes when the proper equipment is used. Compared with, *e.g.*, the direct injection of 500 μl of plasma or urine on a short silica-based pre-column, on-line dialysis has the advantage of a long pre-column lifetime, better ruggedness and the possibility of working in a pulsed mode (see below). Normally more than 200 samples can be injected without the need to change the precolumn and more than 500 samples may be introduced on one membrane.

Pulsed dialysis

If a very low limit of detection is required and the available volume of sample exceeds the volume of the dialysis donor channel, static dialysis of multiple aliquots (pulses) of the sample may be attractive.

Two practical problems arise when this pulsed dialysis mode is used, *viz.*, the increased analysis time and the increased volume of acceptor solvent that is led over the precolumn, which may cause analyte breakthrough. In this study, one to five 470-μl pulses of a standard solution containing the nitrofurans were each subjected to a 3-min static dialysis under variable acceptor flow-rates (*i.e.*, dialysis efficiency) conditions.

The results in Fig. 7 show that at the lowest acceptor flow-rate of 0.18 ml/min

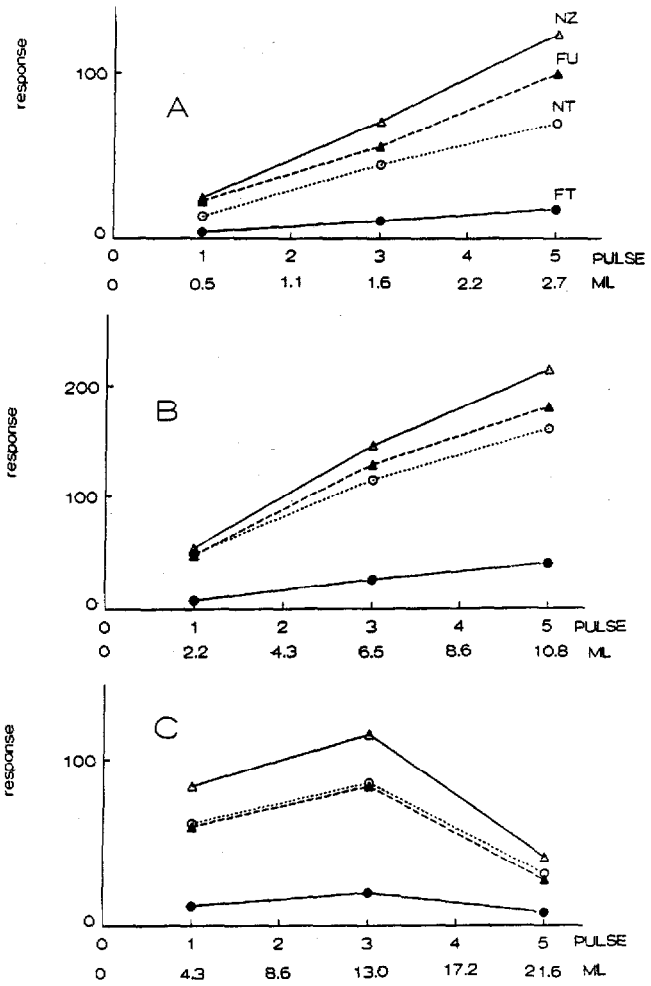

Fig. 7. Plot of the relative UV response (365 nm) obtained when an increasing number of 500-μl pulses of an aqueous nitrofuran standard solution were each dialysed for 3 min in the static mode at an acceptor flow-rate of (A) 0.18, (B) 0.72 and (C) 1.44 ml/min. The number of successive 500-μl pulses and the total acceptor solvent volumes thus applied to the enrichment column are indicated on the abscissa.

the response increases virtually linearly with the number of pulses. At 0.72 ml/min, however, the response obtained after five pulses is 20–35% below the level expected on the basis of the response obtained with one pulse for the three most polar drugs, NZ, FU and NT. This indicates a distinct loss due to breakthrough of these analytes. The apolar furaltadone is still recovered quantitatively when about 11 ml of acceptor solvent are led over the precolumn. Under high dialysis efficiency conditions, *i.e.*, at an acceptor flow-rate of 1.44 ml/min, about 55% of NZ, FU and NT and 45% of FT are lost after three pulses. These results clearly show that, when the sensitivity obtained with one high-efficiency pulse is not sufficient, a compromise should be made between the number of pulses and the dialysis efficiency per pulse, *i.e.*, the acceptor

flow-rate, when all other parameters are held constant. Here, the retention characteristics of the analytes on the precolumn must certainly be taken into account. In addition, one should consider that high acceptor flow-rates combined with large precolumns may generate back-pressure problems.

General remarks on the optimization experiments

In veterinary drug residue analysis, the available amount of sample is generally sufficiently large (10–100 g) to produce an aqueous extract of more than 10 ml. In therapeutic drug monitoring, on the other hand, often only minute amounts of body fluids are available, which makes it necessary in many instances to use the entire sample in the analysis. In practice, the volume of the dialyser donor channel ranges between 100 and 1500 μl. In view, then, of the results of the optimization experiments described above, the CF mode of dialysis cannot be considered optimum for small-sized samples as the combination of a low dialysis efficiency and a small absolute amount of analyte may result in too high limits of detection. Instead, a static dialysis with a high acceptor flow-rate in a dialyser with a donor channel volume just exceeding the sample extract volume should be used. For samples with a volume exceeding the largest dialyser volume available, any of the three dialysis modes (static, pulsed and CF) can be used, depending on the required sample throughput and the required limit of detection.

In order to illustrate the features of the two most attractive dialysis modes (the CF and pulsed modes) experiments were set up to establish the efficiency per unit time and per unit sample volume. The examples depicted in Fig. 8 show that a 3–5-fold increase in response per 1 ml of sample can be obtained by going from CF dialysis to pulsed dialysis at the cost of, however, a 6–10-fold increase in dialysis time. On the other hand, with CF dialysis one can obtain the same overall response as with pulsed dialysis in about half the dialysis time, provided that a 5-fold larger sample volume is

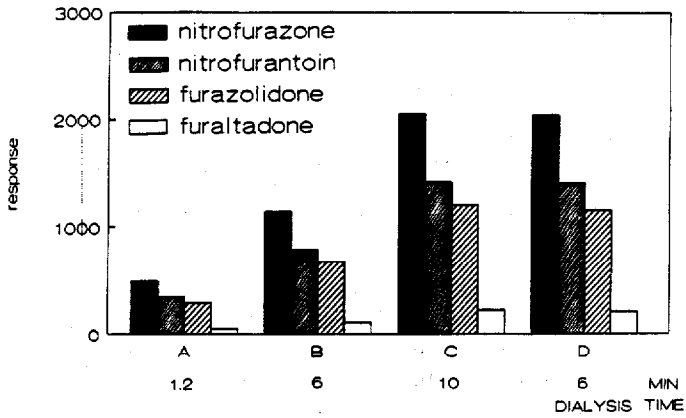

Fig. 8. Plot of the relative UV response (365 nm) obtained when different volumes of an aqueous nitrofuran standard solution were dialysed either in the pulsed static mode or the continuous flow mode. (A) 1 ml in the continuous-flow mode; (B) 1 ml as two 500-μl pulses each with a 3-min static dialysis; (C) 1 ml as two 500-μl pulses each with a 5-min static dialysis; (D) 5 ml in the continuous-flow mode. In all instances the acceptor flow-rate was 0.36 ml/min. The donor flow-rate in the continuous-flow mode was 0.9 ml/min. The total sample dialysis time is given on the abscissa.

TABLE II

SUMMARY OF ASTED CYCLE USED FOR THE DETERMINATION OF NITROFURAN DRUGS IN EDIBLE TISSUES[a]

Time (min)	*Activity*	*Acceptor volume (ml)*
0	Start injection of 1st 750-μl pulse	
1	Start static dialysis	
4	End static dialysis	
	Start application of acceptor solvent on precolumn	1.08
	Start injection of 2nd 750-μl pulse	
5	Start static dialysis	
8	End static dialysis	
	Start application of acceptor solvent on precolumn	2.16
	Start injection of 3rd 750-μl pulse	
12	End 3rd 750-μl pulse	3.24
16	End 4th 750-μl pulse	4.32
20	End 5th 750-μl pulse	5.40
24	End 6th 750-μl pulse[b]	6.48
	Start flushing precolumn	
32	End flushing of precolumn	8.64
	Start backflushing of analytes from precolumn	
37	End of backflushing of analytes	
	Separation of analytes on analytical column	
	Start purging donor channel (10 ml)	
	Start purging acceptor channel (2 ml)	
	Start regeneration of precolumn (2 ml)	
47	Injection of 1st pulse of a new sample	

[a] Extensively described for the 1st and 2nd pulses only.
[b] The 6th pulse consists of 500 μl of the 5th pulse plus the remaining 250 μl of the sample.

injected. Clearly, when the amount of sample, including the maximum amount that can be injected, is critical, pulsed dialysis is the mode of choice. When, on the other hand, a high sample throughput is necessary, CF dialysis should be selected. In all instances, the breakthrough of analytes on the precolumn should be investigated.

Determination of nitrofuran residues in edible products

As an illustration of the practicality of the on-line dialysis–column-switching HPLC system, the trace-level determination of nitrofuran drugs in edible tissues was investigated using the pulsed dialysis mode. On the basis of the results of the optimization studies, the following conditions were selected. Two Gilson dialyser blocks were placed in series, giving a total donor volume of 740 μl and a total acceptor volume of 1300 μl. The 4-ml sample extract was injected as five 750-μl pulses and one 250-μl pulse. The final pulse thus consisted of 500 μl of the already dialysed fifth pulse and the remaining 250 μl of sample extract. The first five pulses were statically dialysed for 3 min at an acceptor flow-rate of 0.36 ml/min, giving a dialysis efficiency of about 30%.

The final pulse was statically dialysed for 9 min at the same acceptor flow-rate. Using this prolonged dialysis time, the last 2 ml of acceptor solvent are essentially free from matrix components, and can be used to flush the precolumn. The total acceptor

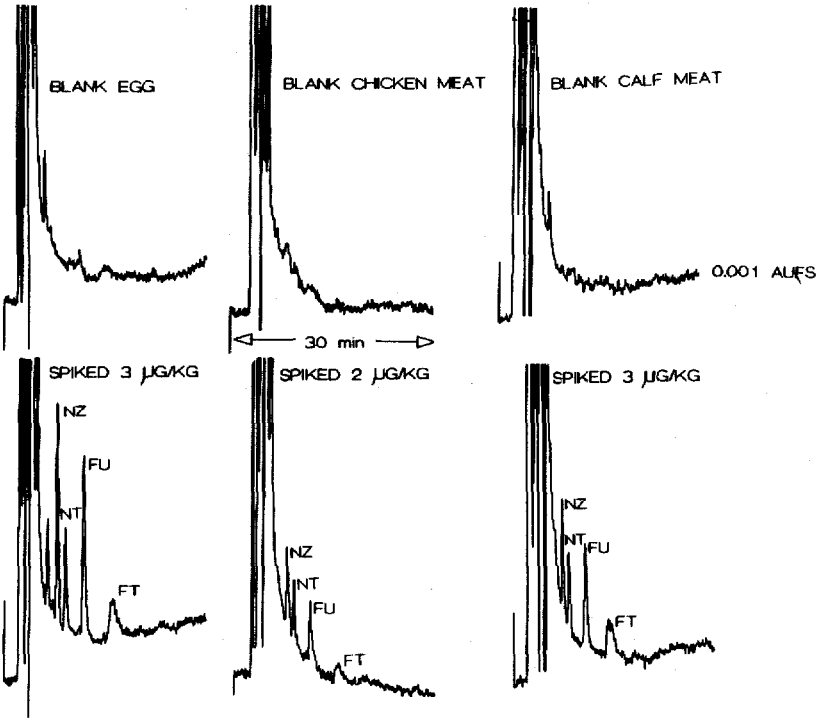

Fig. 9. Chromatograms of blank egg, chicken meat and calf meat samples (upper chromatograms) and corresponding nitrofuran-spiked samples (lower chromatograms). Detection at 365 nm, 0.001 a.u.f.s. For further details, see Experimental and Table II.

volume that was led over the precolumn was about 8.6 ml. After application of this total volume, the pre-column was backflushed for 5 min with HPLC eluent and the enriched analytes were separated on the analytical column. Table II shows a summary of the ASTED cycle.

TABLE III

LIMITS OF DETECTION (LOD) AND RECOVERIES OBTAINED FOR THE NITROFURAN DRUGS IN MEAT (VEAL, CHICKEN) AND EGGS

Drug	*Meat*			*Eggs*		
	LOD (µg/kg)	*Recovery*[a]		*LOD (µg/kg)*	*Recovery*[a]	
		%	*C.V. (%)*		*%*	*C.V. (%)*
Nitrofurazone	2	76	1.7	1	85	4.1
Nitrofurantoin	2	75	2.6	1	76	2.0
Furazolidone	2	89	2.2	1	88	4.8
Furaltadone	5	86	4.0	3	86	6.5

[a] $n = 5$; 5 µg/kg.

Fig. 9 shows some typical chromatograms obtained with blank and spiked egg and meat samples. Based on the chromatograms of blank samples, the limits of detection in egg and meat are calculated to be 1–2 μg/kg for FU, NZ and NT and 3–5 μg/kg for FT (see Table III). In milk samples, NZ and NT could not be accurately determined owing to an unidentified matrix interference. However, the more important drugs FT and FU could be determined at levels above 10 and 5 μg/l, respectively. The procedure was found to be linear between 1 and 100 μg/kg, as was established with spiked meat and egg samples. The response of spiked samples was compared with that of standard solutions subjected to the on-line dialysis–column-switching HPLC procedure. The recovery was found to range between 75 and 90% with low coefficients of variation (Table III). This indicates that with the chosen experimental set-up, the dialysis efficiency and subsequent trace enrichment of the nitrofurans from the crude biological samples are comparable to those of pure aqueous standard solutions. Further, as can be seen in the blank chromatograms, the on-line combination of dialysis and trace enrichment very effectively removes both macromolecular and smaller matrix components having UV absorption at 365 nm, in a fully automated procedure.

CONCLUSIONS

The on-line combination of dialysis and column-switching HPLC is an elegant technique for the fully automated clean-up and trace enrichment of (aqueous extracts of) biological samples. Dialysis efficiencies of 15–85% can be achieved in a 3-min dialysis. The dialyser design is critical with regard to the membrane contact surface area per sample volume. The procedure can either be set up to obtain very low limits of detection by employing a (pulsed) static dialysis, or in a high-speed mode by using continuous-flow dialysis. In practice, the choice for a specific mode will depend on the required sensitivity and the ratio of the available sample (extract) volume to the dialyser volume. Depending on the mode selected, a sample throughout of between 30 and 150 per day can be achieved. In both dialysis modes very reproducible results can be obtained. The practicality of the technique is exemplified by the trace-level determination of four nitrofuran drugs in meat, egg and milk. The only manual sample treatment involved is blending with water followed by centrifugation. Under optimized conditions, the nitrofuran drugs can be determined in about 30 samples per day at a concentration level of 1–5 μg/kg using UV (365 nm) detection. In principle, both the limit of detection and the sample throughput could be substantially improved if a precolumn displaying selectivity towards the drugs would be available and/or a suitable post-column derivatization procedure were to be applied. Further research on these aspects, and also for other veterinary drugs, is planned in the near future.

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