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In this fully automated technique for sample cleanup before chromatographic or other quantitation steps, analytes in body fluids are enriched and semi-purified on a first column. After their selective elution, analytes are "transformed" by admixing appropriate solvents in such a way that they are focused on the top of a second column. By backflush, they are transferred to an analytical liquid-chromatographic column (or simply eluted for quantitation by other techniques). This technique is illustrated by the liquid-chromatographic assay of triamcinolone from a 1-mL urine sample, with ultraviolet detection. Because analytical recovery is almost complete and precision high, no internal standardization is necessary. Interference is eliminated as well as or better than with manual techniques. Chief advantages of this technique are online operation, processing of samples of larger volume, low cost with respect to extraction devices, and nearly universal applicability for exogenous or endogenous compounds of clinical relevance. It potentially may be widely applied.

Additional Keyphrases: sample semi-purification before analysis • economics of laboratory operation

In analyses for low-concentration exogenous or endogenous compounds in human body fluids, sample pretreatment before quantification is still an essential first step in most commonly used analytical techniques such as gas chromatography (1), "high-pressure" liquid chromatography (2), and even radioimmunoe assay (3–5). Increased demand for assay of substances such as drugs in the routine laboratory has motivated the search for technical alternatives that may decrease the labor and time involved and improve assay speed and reliability of the classical manual-extraction techniques. A considerable step forward was the introduction of commercial "minicolumn" techniques based on the extraction of solutes from solid matrices, such as the "Jetube" (6), the "Extrelut" (7–10), the "Sep-pak" (11–13) or the "Chrom Prep" (14) techniques. Meanwhile, these techniques have been further developed to automated or semiautomated versions (e.g., 15). The disadvantages still inherent in these techniques are such that (a) the analyte-containing eluates have to be manually handled for the final quantitation step; (b) in the course of the heterogeneous-phase step (e.g., evaporation of organic solvent), unstable substances may decompose, partly or completely; (c) because reproducibility of analyte recoveries is inadequate, internal standards are necessary; (d) the relatively expensive extraction columns are not reusable; and (e) these techniques are not suited for the "on-line" coupling with automatically operating quantification techniques already established, particularly liquid chromatography.

Such a fully automated device that includes sample cleanup and liquid-chromatographic quantitation has recently been put forward by Roth et al. (16) for drug-monitoring purposes. This technique, based on "column sample enrichment" and "pre-column backflush," however, might be restricted only to very low-volume samples, because high-concentration lipophilic substances such as cholesterol or triglycerides in serum accumulate, during repeated sample injections, on the top of the analytical column, thus strongly and quickly altering the properties of column performance when large-volume samples are used.

Here we describe the principle of a new cleanup procedure that may provide the technological basis for a fully automat ed liquid-chromatographic analyzer for (we believe) all endogenous or exogenous substances that can be measured by liquid chromatography. A prototype assay is presented: the "on-line" liquid-chromatographic estimation of triamcinolone in 1-mL samples of urine. We also compare the results with those for classical extraction devices.

Materials

Chemicals

Triamcinolone was obtained from Sigma, Munich, F.R.G., and [3H]cortisol from New England Nuclear Corp., Dreieich, F.R.G. Methanol, dichloromethane, acetonitrile, fluoroacetic acid, NaOH, HCl, and anhydrous Na2SO4 were analytical-grade compounds from Merck Ltd., Darmstadt, F.R.G. One mole per liter solutions of NaOH, HCl, and trifluoroacetic acid were purified by passing 20-mL portions through Sep-pak C18 cartridges (Waters Associates, Königstein/Taunus, F.R.G.).

Mobile Phases and Eluents

Water used in preparation of eluents was purified by passage through a "Lichro-Prep" column (Merck Ltd.), consisting of 30–60 μm (diameter) particles of octadecyl silica. It was de-gassed under reduced pressure immediately before use. Water used as a polar mobile phase in the analytical liquid-chromatographic step was purified by "on-line" passage through a 40 × 4.6 mm (i.d.) mini-column (Knauer Ltd., Berlin, F.R.G.) packed with Lichro-Prep material. Acetonitrile and methanol were used without further purification. The composition of mobile phases and eluents was as follows: (A) water; (B) alkaline solution, 600 mL of 10 mmol/L NaOH mixed with 400 mL of methanol; (C) acid solution, 600 mL of 10 mmol/L HCl mixed with 400 mL of methanol; (D) organic eluent, 400 mL of acetonitrile mixed with 600 mL of water; (E) wash solution, 200 mL of 50 mmol/L trifluoroacetic acid mixed with 800 mL of acetonitrile; (F) acetonitrile; and (G) aqueous methanol, 600 mL of water mixed with 400 mL of methanol.

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Instrumentation

The hardware of the complete prototype for on-line estimation of the steroid consisted of a sampling unit, a sample pretreatment unit, and a liquid-chromatography unit. A sampler from a Technicon AutoAnalyzer I (S in Figure 1), modified for externally controlled operation, was used for automatic sampling.

The sample-pretreatment unit consisted of a motor-driven low-pressure six-way valve (V1) from Analytical Biochemistry Ltd., Munich, F.R.G.; two piston pumps (P1 and P2) from Eldex Lab. Inc., Menlo Park, CA 94025; pump P1 being a Model A-30-S and pump P2 being a Model B-94; two pneumatically driven high-pressure six-way valves (HV1 and HV2) (Model 7010; Rheodyne Inc., Berkeley, CA 94710); a mixing chamber (MC) from Kontron Ltd., Eching, F.R.G., with an internal volume of 1.1 mL; two pre-columns (PC1 and PC2) [40 × 4.6 mm (i.d.); Knauer Ltd.]. Pre-column PC1 was dry-packed with "PRP I" (20–30 μm) from Hamilton, Bonaduz, Switzerland. Pre-column PC2 was packed with ODS-silica from Shandon Southern Products, Cheshire, U.K. Tubing and fitting devices were from H. Knauer Ltd. Pumps, valves, and the sampler were operated by an electronic controller (time relay) from Izumi Denki Corp., Osaka, Japan.

The analytical-chromatography apparatus (Model 1084 B) from Hewlett-Packard, Böblingen, F.R.G., was equipped with two pumps (P2 and P3), a fixed-wavelength detector (254 nm), and a facility for time-programmed collection of eluate fractions (FC). The analytical column (AC) 250 × 4.6 mm (i.d.), was prepacked with ODS-Hypersil (5 μm; Shandon).

Methods

Extraction Procedures for Triamcinolone Estimation in Urine

"On-line"-sample pretreatment: Figure 1 shows the flow diagram of the final fully automated system for liquid-chromatographic analysis for triamcinolone in urine:

Step 1: Urine is delivered from the sampler (S) via valve V1, pump P1, and valve HV1 onto pre-column PC1 for 1 min, where all lipophilic material is absorbed on the reversed-phase matrix. The flow rate is 1 mL/min.

Step 2: The Teflon sample line is transferred by the sampler from the sample vial into bottle I, containing pure water (solvent A), which then is delivered for 1 min. Via valve V1, pre-column PC1 is washed with solvent B (from bottle II) for 3 min, with solvent C (bottle III) for 2 min, and again with water (bottle I) for 1 min.

Steps 3 and 4: Pump P2 starts to deliver water from bottle VII into the mixing chamber (MC) at a flow rate of 2 mL/min. Then solvent D (bottle IV) is delivered for 1 min, the triamcinolone fraction being eluted from column PC1 into the mixing chamber (MC).

Step 5: Valve HV2 is switched to position II, thus transferring and focusing the fraction of triamcinolone, which has been rendered more polar in the mixing chamber, onto pre-column PC2.

Step 6: Valve HV2 is switched back to position I, connecting PC1 again in line with the analytical column (AC). The analytical chromatography system (pumps P2 and P3) starts gradient chromatography: the focused triamcinolone fraction is eluted by "back-flush" from PC1 and separated on the analytical column (AC). The gradient is run from 20% to 50% of solvent F within 15 min, followed by equilibration at 20% for 5 min. Resolved compounds are quantified by ultraviolet detection. For 3H recovery measurements the 3H-labeled steroid fraction is collected in a collector (FC).

Fig. 1. Flow diagram of the fully automated liquid-chromatographic assay of triamcinolone in urine

Valves HV1 and HV2 are shown in position I. The broken lines indicate position II.

Step 7: Valve VI is switched to solvent E (bottle V), followed by solvent F (VI). Concomitantly, valve HV1 is turned to position II, eluting residual material from pre-column PC1 into waste by "back-flush" purging.

Step 8: Valve VI is switched to solvent A (bottle I), followed by switching valve HV1 to position I, rendering pre-column PC1 ready for adsorption of the following urine sample.

All these operations are time-controlled by the electronic controller in such a manner that steps 7 and 8 as well as 1 to 4 are run concomitantly with chromatographic step 6.

Manual solvent extraction. To 1 mL of a urine pool, with triamcinolone added to give a concentration of 200 μg/L, add 5 mL of dichloromethane. Vortex-mix for 15 s and centrifuge for 5 min. Aspirate the aqueous layer and evaporate the organic layer under a stream of air in a water bath at 60 °C. Reconstitute the dried extract with 150 μL of a water/acetonitrile (80/20 by vol) mixture, transfer to a micro-vial, and subject to chromatography run in the gradient mode outlined above in step 6. In a further experiment, additionally wash the dichloromethane layer with 2 mL of 0.01 mol/L NaOH, followed by a wash with 2 mL of water.

Extrelut extraction. Pipette 1 mL of pooled urine to 0.6 g of "Extrelut" (Merck, Ltd.) packed in a plastic syringe (10). Let the aqueous phase diffuse into the solid matrix for 15 min. Add 5 mL of dichloromethane, dry the eluate under a stream of air, reconstitute with the mobile phase, and subject to liquid chromatography. In another experiment, additionally wash the organic phase with 0.01 mol/L NaOH and water before chromatography.

Sep-pak extraction. Prime "Sep-pak" cartridges (Waters, Ltd.) by passing through 2 mL of methanol followed by 2 mL of water. With a syringe, pass 1 mL of pool urine through the cartridge and wash with 3 mL of water. Elute with 3 mL.
of methanol and evaporate the eluate under a stream of air. Reconstitute the residue in the mobile phase and chromatograph it. In an additional experiment, wash the cartridge with 3 mL of methanol/water (40:60 by vol) before the final methanol elution.

Results and Discussion

Efficiency of Sample-Cleanup Procedures

We used urine to demonstrate the cleanup efficiency of the present procedure, because it generally is especially problematic with respect to eliminating nonspecifically interfering substances. For these comparative studies we used a urine sample consisting of pooled patients' specimens submitted to the routine laboratory. It was supplemented with 200 μg of triamcinolone per liter. Figure 2 depicts the chromatograms obtained by use of classical cleanup procedures. The triamcinolone peak is superimposed upon a considerable nonspecific ultraviolet background of urinary compounds if the crude organic extract is chromatographed (Figure 2, a and c). Additional prewashing with alkaline slightly decreases the nonspecific background in the liquid–liquid extraction techniques (Figure 2, b and d). Similarly, prewashing with a methanolic aqueous phase in the adsorption technique eliminates nonspecific ultraviolet background (Figure 2f) as compared with the chromatogram of the crude methanolic extract (Figure 2e).

Figure 3 illustrates the cleanup efficiency of different prewashing steps when the present on-line device was used. In these experiments, pre-column PCI, loaded with a 1-mL urine sample, was washed for 2 min with the corresponding washing solvents. The triamcinolone fraction was eluted with the organic solvent D positioned in bottle V, focused on pre-column PC2, then analytically chromatographed. Back-flush purging was done only with solvent E (bottle V7). After washing solely with water (bottle I) no triamcinolone peak was discernible within the large, nonspecific ultraviolet background (Figure 3a). The additional washing with methanol and evaporation of the eluate under a stream of air eliminated the nonspecific peak almost completely (Figure 3b). The last washing with water (Figure 3c) was considerably more effective than the one with water alone (Figure 3d).

Fig. 2. Ultraviolet chromatograms of urine extracts after various manual extraction techniques
I. Solvent extraction: crude extract (a) and after alkaline washing (b); II. Extrelut extraction: crude extract (c) and after alkaline washing (d); III. Sep-pak extraction: crude extract (e) and after alkaline washing (f). The chromatographic features are outlined in Methods.

Fig. 3. Ultraviolet chromatograms of urine extracts before and after additional different cleanup steps, with use of the on-line pretreatment device
Same urine pool used here as in Figure 2. Sequence of washings: pure water (a), after additional methanolic water (b), after additional methanolic NaOH (c), after additional methanolic HCl (d).
anolic water (solvent $G$, in bottle II) eliminated some non-specific compounds that are more hydrophilic than the triamcinolone fraction (Figure 3b). The additional washing with alkaline solvent B (in bottle III) transformed phenolic and acid compounds into their anionic forms, and thus they were eluted from the column while neutral substances such as triamcinolone were left on the column (Figure 3c). In separate experiments, varying the duration of alkaline washing, we showed that the duration of this step is critical for complete elimination of phenolic or acid contaminations, obviously depending on the absolute amounts of these substances that are present in urine. The acid washing solvent C (in bottle IV) additionally eliminates basic compounds that are ionized by acids and again leaves neutral substances on the column. It is apparent that, at the end, only neutral substances having a polarity similar to that of triamcinolone remain on pre-column PC1. Our comparison of the various extraction procedures documents that the present on-line version provides an efficiency that is similar or superior to that of the classical techniques. The principle of the cleanup procedure outlined here for neutral substances is similarly applicable for basic or acid substances. For example, the acid form of an acid organic analyte is enriched on the reversed-phase matrix and washed with an organic modifier, leaving only the acid analyte on the matrix. In contrast to neutral substances, the acid analyte is eluted simply by increasing the pH of the eluent. The analyte is thus transformed into its ionic form and is rendered less adsorptive, while basic and neutral compounds remain on the matrix. Furthermore, this principle may also be extended to cleanup procedures for strongly polar substances by using ion-exchange supports in the pre-column PC1, a technique already described for the cleanup of plasma catecholamines (17).

Focusing before Final Quantitation

The efficiency of focusing of the enriched, semi-purified, and selectively eluted steroid fraction from column PC1 onto the top of the second pre-column, PC2, is demonstrated in Figure 4. In one experiment, steroid standards dissolved in 50 $\mu$L of mobile phase were directly injected onto the analytical column (Figure 4a). In the other experiment, steroid standards dissolved in 1 mL of water were transferred onto the column by using the present sample-pre-treatment system (Figure 4b). There was virtually no significant difference as to peak width and resolution. Direct elution of absorbed compounds from pre-column PC1 in the "forward-flush" mode onto the analytical column resulted in considerable zone-spreading of peak shapes.

Analytes can be refocused on a second pre-column by altering the eluent or the chemical nature of analyte itself in the mixing unit. As outlined here for triamcinolone, neutral analytes are rendered more adsorptive by increasing the polarity of the eluent. Similarly, anionic forms of organic acids are transformed to the more adsorptive neutral acids by pH decrease or cationic forms of amines to the more adsorptive basic forms by pH increase. Alternatively, by admixing a reactive solution with the eluent, the analyte is chemically transformed into a more adsorptive and (or) better-detectable derivative ("on-line" pre-column derivatization).

Analytical Recovery

Potential losses of analytes during the complete assay were monitored by measuring recovery of [3H]cortisol eluted in the corresponding fraction from the analytical column. Mean recovery was 93.8% (SD 2.6%, $n = 12$). Evidently steroids are nearly quantitatively extracted on column PC1 and transferred to the analytical column. This efficient extraction capacity of the reversed-phase material we used accords with the findings of others (II–13).

Precision

The reproducibility of the triamcinolone assay was assessed by use of a normal urine sample supplemented with 200 $\mu$g of triamcinolone per liter. For quantitation, the integral of the peak area was used. The coefficients of variation were 3.3% ($n = 15$) for within-day precision and 5.6% ($n = 8$) for day-to-day precision. These data, together with those for recovery, indicate that this method is suitable for operation without an internal standard. As long as the precision of the first pump $P_1$, is warranted, only one calibration run is necessary with the compound to be assayed as external standard, thus eliminating the difficulties often inherent in internal-standardization procedures.

Except for the drug-enrichment system devised by Roth et al. (16), internal standardization is mandatory for almost all other classical extraction techniques.

Specificity, Sensitivity, and Memory Effects

Ultraviolet absorbance is well known to be very non-specific as a detection signal, especially so for assay of low-concentration substances in urine. Using the present procedure—including, if necessary, a longer alkaline wash—we found that the nonspecific ultraviolet background was completely eliminated in nearly all freshly sampled specimens of urine from normal subjects or patients. However, in older urine samples or in urine of patients under high-dosage intensive therapy, considerable amounts of nonspecific absorbances were often still present in the triamcinolone area, which excluded reliable estimation of this steroid. Thus, in four of 32 samples from patients in the intensive-care unit who were being treated with high dosages of antibiotics and glucocorticoids, triamcinolone could not be distinctly assessed, for this reason.

The detection limit (a signal threefold the height of the noise level) was about 5 $\mu$g of triamcinolone per liter for a 1-mL urine sample. There was no measurable memory effect when water was run immediately following a sample containing 500 $\mu$g of triamcinolone per liter.

Fig. 4. Chromatograms of triamcinolone and cortisol standards

Direct injection of 50 $\mu$L with the variable Hewlett-Packard injection device (a) and the on-line pretreatment device enriching steroids from 1 mL of water (b). Amount of each steroid finally injected: 250 ng
Stability of the System

In a series of 300 analyses of 1-mL urine samples, the chromatographic resolution, peak shapes, and recovery were of consistent quality. The limiting factor for stability was the development of dead volumes in pre-column PC2 and the analytical column; however, this was easy to correct by filling up these gaps with the corresponding support material.

Practicability

In the present system, operating without internal standardization, manual manipulations are decreased to only the transfer of the crude centrifuged sample into the automatic sampler. The coupling of the electronically controlled units of the cleanup device and the chromatograph provides unattended operation, even overnight. The interval from sampling to the first result, including the pretreatment and the chromatographic phase, is 40 min. If one is running samples serially, the time for analysis is determined only by the chromatographic time, because sample pretreatment of the following sample is performed concomitantly. Thus, for triamcinolone assay as described here, 48 samples may be handled during one day, if overnight operation is included.

As for versatility, it seems justified to suggest that the principle of the present technique should be applicable to all substances that have been or will be shown to be assessable by liquid-chromatographic techniques.

Advantages of the present sample-pretreatment technique as compared to classical techniques can be summarized as follows:

- Manual operations are almost completely eliminated
- The highly efficient and reproducible extraction allows analysis with external standardization
- Relatively large volumes of body fluids are quickly concentrated, without contaminating analytical columns as compared with the simple backflush technique
- By utilizing the different physicochemical properties of analytes, of eluents, and of support material, the cleanup procedure provides better purification of analytes before their quantification
- The focusing technique provides high chromatographic resolution
- The regeneration of precolumns and the use of inexpensive solvents for sample cleanup provide economical operation as compared with the relatively expensive one-use cartridges
- Decreased use of toxic and highly inflammable organic solvents
- The sample-pretreatment device (steps 1–3) is equally well suited for use as an automatic prepurification and enrichment step before other quantification techniques, such as immunoassays

Because of the versatility allowed by the few solvents required and the simplicity of the microprocessor-controlled operation of the different steps, this technique may find wide application in the future.

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References