

erides (2). In contrast to bilirubin, it is extractable in the nonane phase and interferes with the color produced in the Hantzsch condensation (unpublished observation).

Serum Q may be a representative control serum, but for unknown reasons we obtained large deviating results with three kits. Serum R shows the same behavior as sera N-P.

From this study we conclude that, for interlaboratory standardization and quality control of this determination, only human serum-based standards with a cholesterol concentration ≥ 5 mmol/L can be recommended. Unfortunately, preparation of such a control material is expensive. In certain cases, use of human serum-based material enriched with animal lipoproteins may also be permitted.

When one takes into account the availability of several enzymic kits that give accurate values in the analysis of pooled human serum (1), it is tempting to consider these enzymic methods as alternative reference methods (3), but our results show that the enzymic kits are very sensitive to

matrix effects, so this may be premature.

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Microscale Ultrafiltration Technique for Determining Free Drug in 50- μ L Serum Samples

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This ultrafiltration technique allows determination of free drug in 50 μ L of serum. We ultrafiltered sera containing the following drugs—valproic acid (and its major metabolites), phenobarbital, diazepam, indomethacin, phenytoin, furosemide, and chloramphenicol—using both the commercially available micropartition system (MPS-1, Amicon), which requires a 200- μ L sample, and our modified micro system, which requires only 50 μ L. The value for the free fraction of each drug obtained in the two experiments agreed well. The smaller sample requirement makes the micro method particularly suited for pediatric samples and studies on small laboratory animals.

Additional Keyphrases: *pediatric and veterinary application · drug assay*

The techniques most commonly used for determination of free drug in serum are equilibrium dialysis and ultrafiltration (1, 2). Both methods, in their present form, suffer from a common drawback: about 150 μ L of serum is required for a single determination. Although not a problem for determinations on samples from adult patients, this is a problem when samples from newborns or infants are to be investigated or pharmacokinetic studies are to be performed on experimental animals, where repeated sampling in short intervals is often necessary (3). Ultrafiltration (UF) is a fast technique, and it can be routinely applied to the determination of free drug in samples of at least 150 μ L by using a commercially available system (MPS-1, Amicon) (4, 5). We

describe here a modification of this system that requires only 50 μ L of serum for one determination.

Materials and Methods

Chemicals. ¹⁴C-labeled analogs of furosemide, diazepam, phenobarbital, and chloramphenicol were obtained from Amersham Büchler, Braunschweig, F.R.G.; the ¹⁴C-labeled analogs of phenytoin and indomethacin from New England Nuclear, Dreieich, F.R.G.; and valproic acid (VPA), 2-propyl-2-pentenoic acid (2-en-VPA), and 2-propyl-4-pentenoic acid (4-en-VPA) from Desitin-Werke Carl Klinke, Hamburg, F.R.G.

Serum was obtained from volunteers who were not receiving any drug treatment.

Ultrafiltration devices. (a) Commercial version: We used the commercially available MPS-1 system from Amicon, Danvers, MA (Figure 1A), which is described as a micropartition system for separation of free from protein-bound substances for sample volumes of 0.15 to 1.0 mL (Amicon publication 460G).

(b) Modified version: In our own version (Figure 1B) the following parts of the original version (Figure 1A) were modified: For sample tube (2) we substituted a modified tube (a), which is of the same inner diameter but shorter. At its base it is designed (Figure 1C) to fit to an O-ring (b) smaller in diameter than that used in the original system (3). The O-ring (b), inner diameter 6.5 mm, determines the area on the filter membrane that is actually available for the filtration. The commercial filtrate tube (7) was altered to serve as a funnel (c) for collection of the ultrafiltrate in a standard disposable 1.5-mL Eppendorf vial (d).

Binding experiments. A known amount of each drug was added to 2.5 mL of drug-free human serum and the samples were additionally supplemented with a small amount of ¹⁴C-

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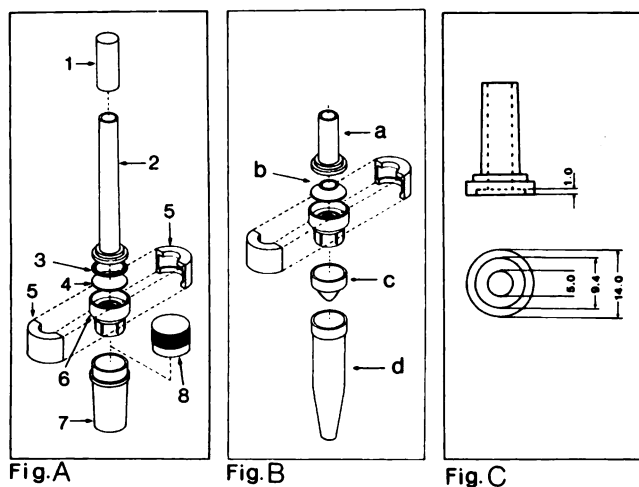


Fig. 1A. Amicon MPS-1 system (commercial version)

1) cap; 2) sample tube; 3) O-ring; 4) YMB-membrane (membrane diameter is 14 mm); 5) clip; 6) filterholder; 7) filtrate collection vial; 8) cap.

B: Modified micro version

a) sample tube*; b) O-ring (o.d. 9.5 mm, i.d. 6.5 mm); c) funnel*; d) disposable 1.5-mL Eppendorf microvial. *Made in our workshop

C: Detailed view of sample tube (a)

Dimensions in mm

labeled analogs. In each case the concentrations represented therapeutic drug concentrations. The fortified serum samples were allowed to equilibrate at 37 °C for 2 h. For each drug we centrifuged eight samples, using the commercial version with a sample volume of 200 µL, and eight more samples, using the modified version with a sample volume of 50 µL. The samples were transferred into the sample tubes (2 and a in Figure 1) and the assembled filtration devices were centrifuged in a Sorvall RC-513 centrifuge fitted with a fixed-angle rotor at 4500 rpm for 15 min; the temperature was maintained at 37 °C during centrifugation. Radioactivity in the ultrafiltrates as well as the serum samples was counted in a liquid scintillation counter (LKB 1217, Rackbeta), from which we calculated the free-fraction values.

In the case of VPA, 4-en-VPA, and 2-en-VPA gas chromatographic-mass spectrometric monitoring (6) was used. Ultrafiltration was as described before; however, we found that the membranes (YMB, Amicon) contain glycerol, which interferes with the analysis of 2-en-VPA. Therefore, the membranes were repeatedly washed with distilled water and centrifuged under the same conditions as outlined above, water being substituted for serum. After this pre-treatment, filtration of the samples was performed as described before.

Depending on the sample volume, we obtained between 130 and 150 µL of ultrafiltrate with the commercially available method (200 µL of serum) and between 15 and 20 µL with the microversion (50 µL of serum). For analysis of the ultrafiltrate from the commercial version we took an aliquot and either counted its radioactivity in the scintillation counter or processed it for the gas chromatographic-mass spectrometric assay. In the micromethod only small volumes of ultrafiltrate were obtained. When radiolabeled compounds were used, we transferred the droplet of ultrafiltrate into a scintillation vial, which we weighed before and after the transfer, to determine the exact amount of ultrafiltrate. For the gas chromatographic-mass spectrometric

assay, the Eppendorf vial (d) was weighed before and after ultrafiltration for an accurate determination of the sample size. The extraction step could then be done in the same vial, thus minimizing sample losses.

Results and Discussion

The free fraction of individual drugs as determined in both ultrafiltrates agreed well (Table 1).

Some drugs of interest may bind to the filtration membrane. This can be easily tested by filtration of a drug-containing sample in buffer. If no absorption occurs, the concentration of the drug before and after ultrafiltration should be the same. In a recent communication (7) we reported the binding of diazepam to the filtration membrane. In the present paper the free fraction of diazepam was determined both ways and a comparison of the conventional and the micromethod led to the same results (Table 1). Evidently, even though the sample volume and the membrane area available for the filtration are different in the micromethod and the conventional method, the absorption effects are the same.

Table 1. Values Obtained for the Free-Drug Fraction in Ultrafiltrates from the MPS-1 System (200-µL Samples) and the Present System (50-µL Samples).

Compound	Concn in serum, mg/L	Free drug, %, mean (and SD)*	
		MPS-1	Micro version
Valproic acid	60	9.5 (0.78)	9.0 (0.81)
4-en-VPA	20	10.8 (1.23)	11.4 (1.03)
2-en-VPA	20	0.48 (0.05)	0.52 (0.08)
Phenobarbital	20	54.7 (0.18)	56.0 (0.74)
Diazepam	0.5	1.85 (0.06)	1.89 (0.09)
Indomethacin	3	4.40 (0.13)	4.57 (0.09)
Phenytoin	15	11.7 (0.58)	10.9 (0.63)
Furosemide	3	1.33 (0.08)	1.20 (0.08)
Chloramphenicol	5	40.4 (1.6)	41.3 (1.7)

* Eight aliquots of the same serum sample were analyzed for each drug.

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