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Note

Sensitive high-performance liquid chromatographic determination of amiloride in human plasma by direct injection with pre-column enrichment

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Amiloride is an antihypertensive drug possessing both potassium-sparing and diuretic activity. Although the drug has been used clinically since 1965, the first chromatographic method with sufficient sensitivity for pharmacokinetic studies was published in 1985 by Vincek et al. [1]. Very low levels of amiloride present in serum after therapeutic doses may be the reason for that. Vincek et al. [1] used reversed-phase high-performance liquid chromatography, fluorescence detection and prechromatographic sample clean-up on a silica extraction cartridge. The sensitivity of this method was 1 ng/ml. More recently an HPLC method with a sensitivity of 0.5 ng/ml, using only a protein precipitation step prior to injection, has been published [2].

This paper describes an HPLC method with a sensitivity below 0.2 ng/ml of serum obtained by direct injection of serum into the chromatograph equipped with an enrichment column, instead of the injection loop, for sample concentration and clean-up.

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EXPERIMENTAL

Apparatus

The HPLC system consisted of a Kontron (Zürich, Switzerland) LC 410 pump and SFM 23/B spectrofluorometric detector (excitation at 372 nm and emission at 420 nm). The injector was a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 equipped with a 14 mm × 3.9 mm I.D. LiChroprep (Merck, Darmstadt, F.R.G.) RP-18 (40–63 μm) enrichment column in place of the injection loop as described by Nielsen [3]. This column was renewed and preconditioned every day by injection of two serum blanks, and it lasted for ca. 100 samples.

Chromatography

The column (30 cm × 3.9 mm I.D.) was pre-packed with μBondapak C_{18} (10 μm) and the protection column (23 mm × 3.9 mm I.D.) was packed with Bondapak C_{18}/Corasil (37–50 μm), both from Waters (Milford, MA, U.S.A.).

The mobile phase was acetonitrile–0.01 *M* phosphate buffer, pH 3 (8:92). Acetonitrile was filtered through a 0.5-μm FH-Millipore filter and the phosphate buffer through a 0.5-μm HA-Millipore filter. The mobile phase was degassed ultrasonically.

The flow-rate was 2 ml/min, the column temperature was 40°C and the pressure was ca. 100 bar.

Reagents and glassware

Distilled water was used throughout. Acetonitrile 'zur Rückstandanalyse' was from Merck. Methanol and KH_2PO_4 were of analytical grade from Merck. Moduretic®, MSD tablets (Batch NE 3071) containing 5 mg of amiloride and 50 mg of hydrochlorothiazide were used for dosing to experimental subjects.

Glass test-tubes were used for blood sampling and storage of serum at −20°C.

Procedure

Serum (1 ml) was added to 2 ml of water. This mixture was treated for ca. 5 s on a whirlimixer and stored cold and protected against light until injection. Every injection onto the enrichment column consisted of 1 ml of methanol, 1 ml of water, 1 ml of sample and 1 ml of water. This enrichment procedure took ca. 1 min. Then the sample was loaded onto the chromatographic column. The retention time for amiloride was ca. 5.8 min.

Standard solutions of amiloride in serum were treated and analysed simultaneously with samples. The sample concentration was calculated on the basis of the peak height of amiloride, by reference to the standard curve (blank and 1, 5, 10 and 20 ng/ml) obtained by linear regression.

Testing of the analytical procedure

The accuracy, precision and linearity of the method were determined by using spiked samples of human serum analysed at random. Total recovery of amiloride was determined as the response of analysed samples, relative to the response of solutions, directly injected.

The stability of samples was tested from spiked human serum and from samples obtained after administration of Moduretic tablets. The samples could be stored at $-20°C$ for more than two months.

RESULTS AND DISCUSSION

The analytical procedure for amiloride in serum was found to be accurate, precise and linear (Table I). The accuracy was 100% (range 96–105%), calculated as the percentage found on the basis of the linear standard curve. The precision, expressed as the relative standard deviation (R.S.D.) was 6% (range 5.0–6.8%). The analytical procedure was linear in the studied concentration range, as the deviation of accuracy from 100% was smaller than 5% and smaller than the precision at all concentration levels tested. The regression line correlation, intercept and slope were 0.998, 0.029 and 0.0174, respectively. Total recovery for amiloride was ca. 85%. The blank value was smaller than the baseline noise (Fig. 1).

Direct injection of serum with pre-column enrichment has been described by Voelter et al. [4]. It is an easy alternative to 'classical' sample preparation by solvent extraction or sample clean-up on an extraction cartridge, but like these methods it has to be optimized to obtain high recovery and reproducibility. Total recovery for water spiked with amiloride (1–20 ng/ml) was ca. 100% (S.D. 6%) without special treatment of the enrichment column. The corresponding recovery from serum was ca. 50% and variable, which was also the case for serum diluted with water (1:9). Dilution of serum with 10–20% ace-

TABLE I

ACCURACY AND PRECISION OF THE ANALYTICAL PROCEDURE FOR AMILORIDE

Spiked human serum was used for six determinations at each level.

Added (ng/ml)	Found (ng/ml)	Accuracy (found, %)	Precision (R.S.D., %)
0	0	–	–
1.00	1.05	105	5.0
4.9	4.8	98	6.8
9.5	9.1	96	5.6
18.2	18.2	100	6.3
Mean		100	6.0

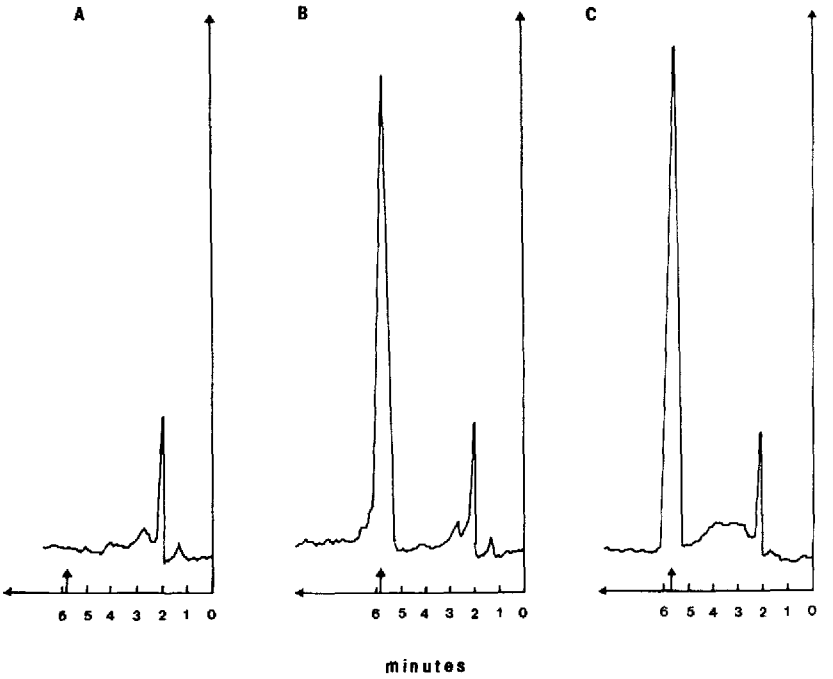

Fig. 1. Typical chromatograms from (A) blank serum, (B) serum spiked with 10 ng/ml amiloride and (C) serum from an experimental subject 5 h after ingestion of 5 mg of amiloride and 50 mg of hydrochlorothiazide as a Moduretic tablet.

tonitrile, as described by Nielsen [3], reduced the recovery considerably. Flushing with 1% ammonium acetate [5] increased the recovery to ca. 75%. However, reconditioning of the enrichment column with 1 ml of methanol before flushing with 1 ml of water, prior to every injection of serum samples, resulted in a total recovery of ca. 85%. To obtain this recovery it was necessary to renew and stabilize the enrichment column every day by initial injection of two serum blanks. The contamination of the chromatographic column was remarkably low, and the column could be rinsed with methanol.

The detection limit of ca. 0.5 ng/ml depends on the enrichment factor, as no blank was observed. For example, injection of 1 ml of undiluted serum resulted in a limit of 0.2 ng/ml, which is five times lower than the limit described by Vincek et al. [1]. Dilution of serum samples with water (1:2) was preferred for routine work, as the lifetime of the enrichment column may thus be increased (up to 100 samples).

Possible interference from resampling metabolites is not likely, as there are no reported metabolites of amiloride [6]. Hydrochlorothiazide, which is often combined with amiloride, does not interfere in the chromatogram (Fig. 1), because it has about twice as long a retention time and does not emit fluorescent light.

The method is rapid and suitable for pharmacokinetic studies requiring high sensitivity. For clinical use the selectivity has to be studied with respect to locally used drugs that are normally co-administered.

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