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Note

Automated column liquid chromatographic determination of polythiazide in human serum

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Polythiazide is a diuretic that is frequently used in the management of hypertension, either alone or in combination with antihypertensive drugs such as prazosin [1]. Since the dosage of polythiazide is relatively low (0.5–5 mg), a very sensitive methodology is necessary for the quantification of drug levels in human serum, which is required for pharmacokinetic or compliance purposes.

The methods described hitherto are sufficiently sensitive [1,2], but require large volumes of serum, are very sophisticated and are scarcely suited for routine purposes. Using the instrumental settings of an automatic liquid chromatographic (LC) analyser previously applied for other analytes [3,4], we have devised a fully automatic procedure for the assessment of polythiazide in serum.

EXPERIMENTAL

Chemicals and solvents

Polythiazide was obtained from PFB (Berlin, F.R.G.); other reagents were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

The following solvents were used for sample clean-up and chromatography: methanol, acetonitrile, deionized water, 20 m*M* lithium hydroxide, 20 m*M* citric acid (CA), 20 m*M* Tris acetate and 50 m*M* trifluoroacetic acid (TFA). The solvents were degassed by purging with helium, then freshly purified on-line as follows: the aqueous solutions by on-line passage through Sep-Pak C_{18} cartridges (Waters Assoc., Königstein/Taunus, F.R.G.); lithium hydroxide by passage through a cartridge packed with 10-μm polystyrene–divinylbenzene copolymer (PRP-1®) particles (Hamilton, Reno, NV, U.S.A.); and methanol and aceto-

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nitrile by passage through cartidges packed with 10-μm alumina particles (Macherey-Nagel, Düren, F.R.G.). Mixtures of aqueous and organic solvents, as well as of buffer solutions, were on-line prepared by the ternary mixing devices of an automatic LC analyser [3].

Instrumentation

The automated LC analyser is identical with the system that is already used for the determination of other analytes [3,4] and has been described in detail elsewhere [4].

Procedure

The following analytical steps are time-controlled by the electronic controller in such a manner that steps 1–5, as well as 7 and 8, are run concomitantly with the chromatographic step 6. All UV-absorbing material that was not loaded onto the analytical column was monitored by a second detector II set at 269 nm, the absorbance maximum of the polythiazide molecule.

Step 1: 1 ml of serum mixed with 0.5 ml of 1 *M* CA is transferred from the sampler into the sampling loop of the sampling unit; concomitantly, column 1 is equilibrated with a CA–lithium hydroxide buffer (pH 3) prepared by mixing 790 ml of CA and 210 ml of lithium hydroxide.

Step 2: the loop is switched into the analytical line and the sample is transferred onto column 1; all lipophilic compounds are adsorbed on column 1, polar compounds are eluted.

Step 3: a mixture of a pH 7 buffer (Tris acetate–lithium hydroxide) and methanol (58:42, v/v) is delivered by pump P1 at a flow-rate of 2.5 ml/min; substances more polar than polythiazide are eluted from column 1 into the waste.

Step 4: a mixture of pH 11 buffer (CA–lithium hydroxide) and methanol (42:58, v/v) is delivered at a flow-rate of 1.3 ml/min; polythiazide is eluted into a mixing chamber.

Step 5: TFA (flow-rate 2.4 ml/min) is concomitantly delivered into the mixing chamber; polythiazide is focused onto the top of column 2.

Step 6: column 2 is switched into the line with the analytical column; a gradient is run from 20 to 58% of acetonitrile in TFA; the prepurified, focused fraction is chromatographed on the analytical column, and separated polythiazide is quantified at 269 nm in detector I.

Step 7: in back-flush mode, acetonitrile–TFA (80:20, v/v) is delivered through column 1; all residual material more lipophilic than the polythiazide-containing fraction is eluted into the waste.

Step 8: pure Tris acetate is delivered through column 1; the matrix of column 1 is equilibrated and prepared ready for adsorption of the next sample.

The retention behaviour of polythiazide as a function of pH was studied in a chromatographic system using a gradient from 20 to 80% of methanol. The different pH values of the buffer were obtained by mixing appropriate volumes of 0.02 *M* CA or 0.02 *M* Tris acetate with 0.02 *M* lithium hydroxide. Column 1, packed with PRP-1, was used as solid phase, the UV absorbance of the eluate was monitored in detector II at 269 nm.

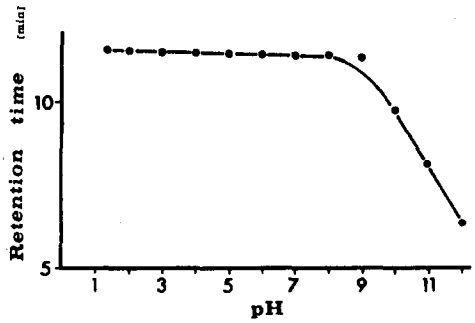

Fig. 1. Retention of polythiazide on PRP-1 as a function of pH. Conditions: 60×4 mm I.D. column, 10-μm PRP-1 phase, with a 20 to 80% gradient of methanol in buffer; flow-rate, 2.5 ml/min.

RESULTS AND DISCUSSION

Procedural variables

Clean-up and chromatographic conditions. Polythiazide is a weak acid. Control of the eluent pH, therefore, can convert this molecule from a neutral into an anionic species, and vice versa. Correspondingly, the plot of the retention time versus the pH shows an inflection point at pH ca. 10.5, the pK_a of the acid molecule (Fig. 1). For preconcentrating and cleaning-up polythiazide, we used a buffer component of pH 7. An acceleration and rapid elution of the polythiazide molecule into the mixing chamber is achieved by changing the pH to 11. Under these conditions, the elution of basic and neutral compounds remains slow, and hence they are retained on the column 1. The accelerated, eluted polythiazide anion is again converted into the neutral form in the mixing chamber by changing the pH to acid conditions. Thus, the neutral form with a higher adsorbitivity is focused on the top of column 2. By switching this column in line with the analytical column, this neutral species is effectively separated from substances that have very similar chromatographic and physicochemical behaviour to polythiazide.

Efficiency of sample clean-up. The efficiency of the sample clean-up procedure for elimination of non-specific, UV-absorbing chromogens was studied in a normal serum sample. When the complete clean-up procedure is used, only a few non-interfering peaks are discernible in the final chromatogram (Fig. 2a). The corresponding chromatogram from the same serum sample spiked with 10 ng of polythiazide per ml (Fig. 2b) demonstrates a clear resolution of the drug from the matrix background. Chromatograms obtained from serum samples of twelve in-patients were clean, similar to that shown in Fig. 2a.

Procedural losses. To investigate the potential loss of polythiazide during the complete multistep analytical procedure, a 1-ml water sample containing 10 mg/l polythiazide was assayed. UV absorbance was monitored at the same time with both detectors. Detector II (limit of detection ca. 60 ng) detected no UV absorbance, thus indicating that polythiazide was almost quantitatively transferred to the analytical column. During routine analyses, a check for potential analytical losses like this was done every fiftieth sample.

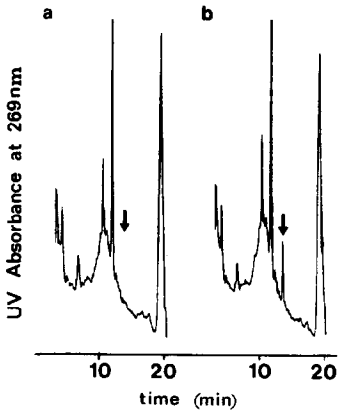

Fig. 2. Chromatograms of a blank serum sample run by the present method (a) and of the same sample spiked with 10 ng/ml polythiazide (b). Arrow indicates elution time of polythiazide.

Stability and quality of the chromatographic system. For 200 serum samples that had been analysed with the same instrument settings, the chromatographic resolution and peak shape were of consistent quality, as was the recovery of 20 ng/ml polythiazide in an aqueous sample. There was no significant alteration of the retention time.

Memory effect. There was a memory effect of 0.2%, when pure water was analysed immediately following a sample containing 10 μg/ml polythiazide.

Analytical variables

Standard curve and sensitivity. A standard curve for external calibration was set up in a serum sample, the concentrations ranging from 2 to 50 ng/ml. A linear calibration curve over the whole range was obtained when the peak integral was evaluated. The detection limit (a signal three-fold the height of the noise level) was ca. 0.5 ng/ml. Unknowns were estimated by their peak integral and the response factor from the calibration curve. This response factor was rechecked every twentieth sample by assaying a sample spiked with 20 ng/ml polythiazide.

Precision. Precision was assessed by replicate analyses ($n=15$) of normal serum samples spiked with 20 and 5 ng/ml polythiazide. Coefficients of variation were 3.2 and 18.5%, respectively.

Recovery. Analytical recovery was determined by comparing the appropriate peak integral of a water and a serum sample, each spiked with 50 ng/ml polythiazide. There was no significant difference.

Selectivity. Fifteen serum samples of in-patients were assayed. There was no UV-absorbing peak eluting with a retention time similar to polythiazide.

Practicability. The described method was applied to large numbers of blood plasma specimens from a clinical study in which polythiazide was administered in 0.5- and 2-mg dosages. Whereas polythiazide levels were readily measurable in the 2-mg experiments, levels of polythiazide were scattered around the detection limit of the method when a single 0.5-mg dosage was administered.

In previously described methods [1,2], large amounts of serum, e.g. 5 ml in ref.

1, are necessary to provide a sensitivity similar to that obtained with the present method. For pharmacokinetic studies, such high amounts of serum are ethically and experimentally not justifiable.

The practicability of the present automated method for the estimation of polythiazide provides further distinct advantages over earlier ones, because: (1) the complete assay is fully automated, thus eliminating any manual extraction or evaporation steps; (2) the analyte is kept in solution throughout the complete assay, thus avoiding potential loss or decompositions; (3) procedural losses of polythiazide throughout the assay are negligible and because of the good precision only a single external calibration is necessary for evaluation of results; and (4) the regeneration of pre-columns and the use of inexpensive solvents renders the method relatively economical.

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