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Report

Column-Switching High-Performance Liquid Chromatographic (HPLC) Determination of Hydrochlorothiazide in Rat, Dog, and Human Plasma

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A fully automated HPLC assay for hydrochlorothiazide in plasma has been developed using a column-switching technique. The method involves direct injection of plasma to the extraction column for sample cleanup followed by switching onto the analytical column. Good precision, accuracy, and linearity were obtained over a range of 25 to 2000 ng/ml in rat, dog, and human plasma. The column-switching method has also been validated by comparison with a conventional HPLC method requiring a cumbersome plasma extraction procedure. Since the method is simple, rapid, and reproducible, it is useful for determination of hydrochlorothiazide levels in animal and human plasma.

KEY WORDS: column-switching high-performance liquid chromatography (HPLC); hydrochlorothiazide.

INTRODUCTION

Hydrochlorothiazide (HCTZ) is a potent diuretic of low toxicity and is widely used in combination with cardiovascular drugs for treatment of hypertension (1–4). HCTZ causes increased elimination of water, which lowers blood volume and reduces blood pressure.

A number of HPLC methods (5–13) have been reported in the last 10 years for assay of HCTZ concentration in biological fluids. These conventional methods require cumbersome extraction procedures which are labor-intensive, are costly in solvent consumption, and tend to introduce imprecision. Therefore, the method involving direct plasma injection onto the HPLC column simplifies the assay and enables full automation.

Two approaches involving direct injection of biological fluids onto the HPLC column have been recently pursued and gained increasing attention. One is injection of serum directly onto the internal surface reversed-phase column for immediate analysis (14,16), and the other is injection of plasma directly onto the short extraction column followed by elution onto the conventional analytical column for further analysis (14,17–21), which is controlled by a column-switching device. These two new methods have created a new dimension in the HPLC determination of biological fluids by eliminating the need for liquid–liquid and the conventional solid-phase extractions and the need for robot operation.

To facilitate toxicological and pharmacokinetic studies, we report here a new HPLC method for the determination of HCTZ in plasma using a column-switching technique. In this HPLC procedure, plasma samples were delivered to the extraction column for sample cleanup, followed by elution onto the analytical column for further separation and detection. This new method provides a high degree of sensitivity and reproducibility and a short analysis time.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile and methanol of HPLC grade were obtained from Burdick and Jackson (Muskegon, MI). Hydrochlorothiazide (HCTZ) and hydroflumethazide (HFTZ) (Fig. 1) were from Sigma Chemical Co (St Louis, MO). Sodium phosphate (monobasic) and glacial acetic acid were purchased from Mallinckrodt Co. (Paris, KY). Drug-free plasma from healthy volunteers was obtained from Biological Specialty Co. (Lansdale, PA).

Standard Preparations

Stock solutions of 1.0 mg/ml were prepared for both HCTZ and the internal standard, HFTZ, by dissolving 25 mg in 25 ml methanol and stored at −20°C. Stock solutions were diluted 10 times with 10 m*M* phosphate buffer, pH 7.2, to

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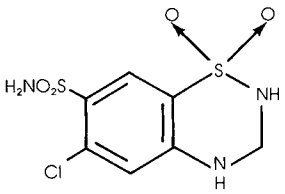
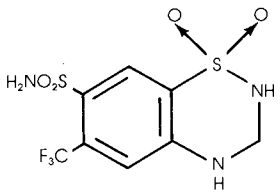


HCTZ



HFTZ
(Internal Standard)

Fig. 1. Chemical structures of hydrochlorothiazide, HCTZ, and the internal standard, hydroflumethazide, HFTZ.

give a concentration of 100 μg/ml, from which plasma standards were prepared by further dilution with control plasma. A working internal standard solution at 1500 ng/ml was prepared by dilution with 10 m*M* phosphate buffer, pH 7.2.

Sample Processing

Frozen control rat, dog, and human plasma samples were first allowed to thaw at room temperature, followed by centrifugation at 3000 rpm for 10 min to obtain particulate-free plasma. For the plasma samples from rats and dogs dosed with HCTZ, an aliquot of 200 to 300 μl thawed plasma from each sample was pipetted into the Eppendorf tubes and centrifuged for 10 min. One hundred fifty microliters of the standards or plasma samples was mixed with 150 μl of the internal standard in an Eppendorf tube. One hundred to 200 μl of the resulting mixtures was injected into the HPLC with the Waters autosampler (Model 700 Satellite WISP). Buffering plasma samples with phosphate at pH 7.2 was found to be helpful in retaining HCTZ and HFTZ on the extraction column, increasing the stability of the plasma sample, and reducing the viscosity of the plasma sample, thus preventing column clogging and prolonging the life span of the extraction column.

Columns and Mobile Phases

The HPLC column system consisted of extraction and analytical columns. The optimal condition for the extraction column was found to require two pieces of microBondapak C_{18} Guard-Pak (Waters Associates), which were used to retain HCTZ and HFTZ until elution with the mobile phase as described below. The Guard-Paks are short (0.4 cm) and inexpensive and incorporate a radial distribution device that spreads the injected sample over the frit surface. This allows the retained compounds to adhere to a narrow band and also reduces the likelihood the frits will become clogged after a number of plasma injections (21). A Hypersil ODS column (5 μm, 4.6 × 250 mm) (Alltech Associates, Deerfield, IL) was used to analyze the HCTZ level in the plasma sample after elution from the extraction column. The guard column was a microBondapak C_{18} Guard-Pak. After 50 to 100 injections, the extraction column was discarded, while the analytical column was extensively washed with a complete cycle of methanol and methanol:chloroform mixture (50:50).

There were two mobile phases, weak (A) and strong (B). Mobile phase A was 10 m*M* phosphate buffer, pH 7.2. It was used to deliver samples to the extraction column. Mobile phase B consisted of 20% acetonitrile, 1% glacial acetic acid, and 79% water, which was used to elute the retained HCTZ and HFTZ onto the analytical column. The mobile phases were degassed with helium prior to use.

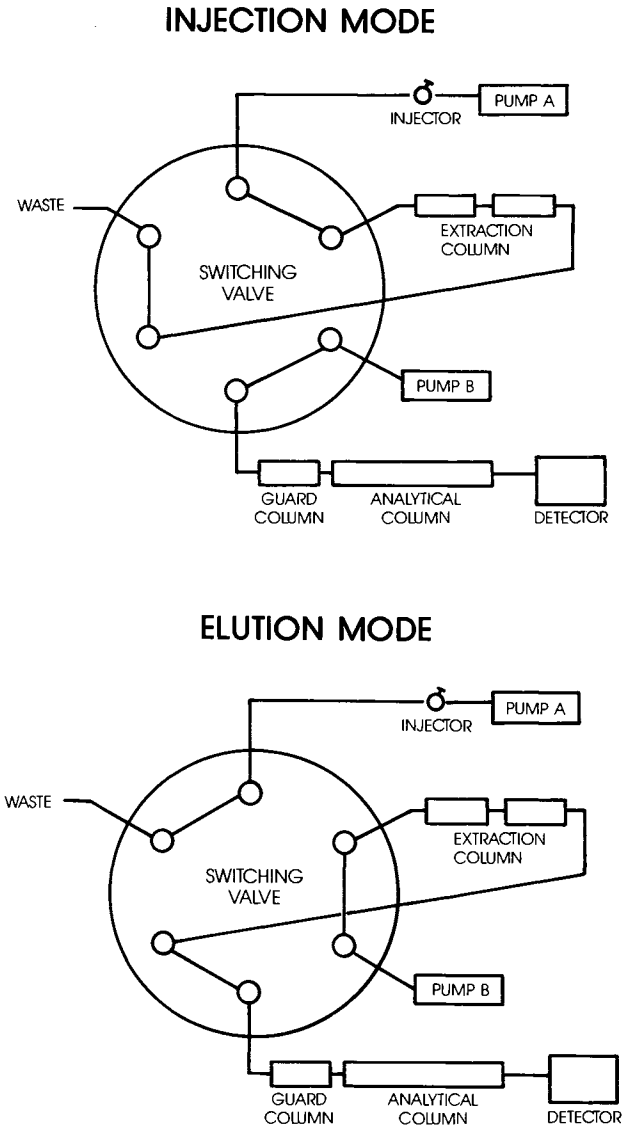


Fig. 2. Schematic representation of column-switching HPLC operation.

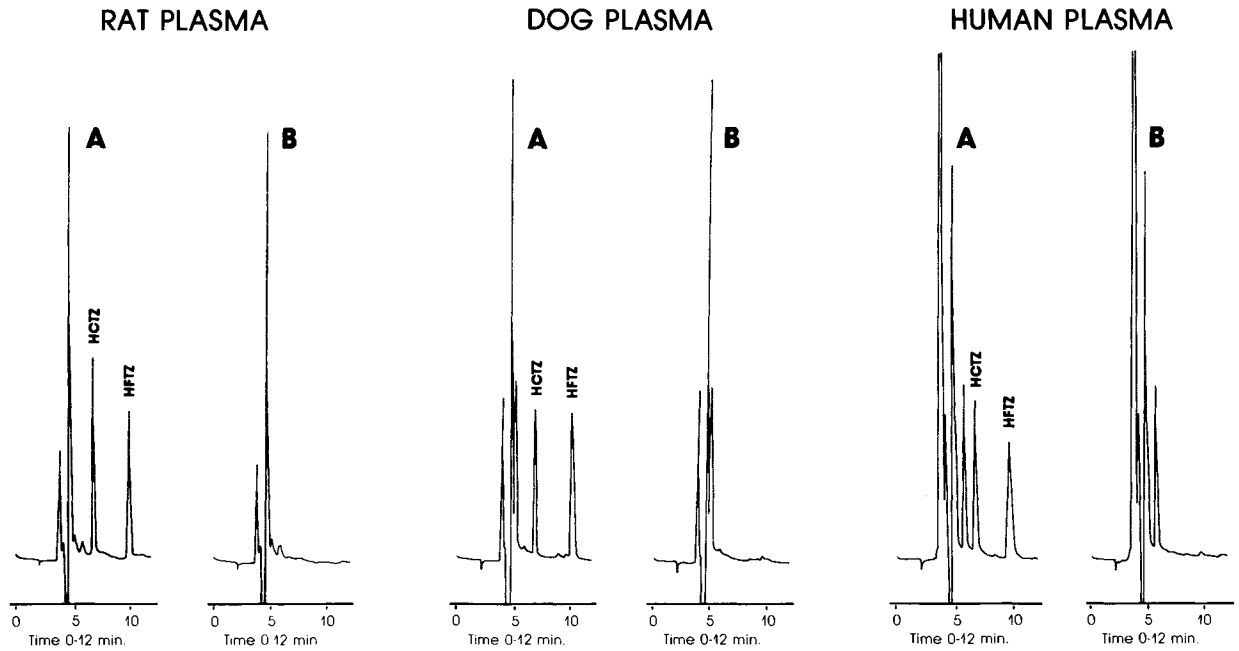


Fig. 3. Typical representative chromatograms from rat, dog, and human plasma spiked with HCTZ (500 ng/ml) and HFTZ (750 ng/ml) (A) and the corresponding control plasma (B).

Operation of Column-Switching Chromatographic System

A schematic of the column-switching HPLC system is illustrated in Fig. 2. Pump A (Waters Model 510) and pump B (Waters 600 Multisolvent Delivery System) were used to deliver mobile phases A (weak) and B (strong), respectively. The flow rates for both mobile phases were set at 1.5 ml/min. The switching valve allowed the mobile phases to follow one of two paths during injection or elution modes. An electrically actuated 10-port Autochrom switching valve (Ann Arbor, MI) was used. It should be noted here that only six ports of the valve were utilized in the present study. All operations were carried out at room temperature.

In the injection mode, the sample was directed onto the extraction column where HCTZ and HFTZ were retained, while unwanted components passed into the waste by mobile phase A. Two minutes after sample injection, the switching valve was automatically rotated to the elution mode. The rotation allowed the mobile phase B to flow through the extraction column and elute HCTZ and HFTZ onto the analytical column for further separation and quantitation. The elution mode lasted 2 min to ensure complete elution of HCTZ and HFTZ. Thereafter, the valve was rotated back to the injection mode, where the extraction column was reequilibrated with the mobile phase A for 8 min while HCTZ and HFTZ underwent chromatographic separation and quantitation. The total run time for an injection of plasma sample was 12 min. The time events of valve switching were controlled by the Waters 600E system controller and the NEC Power Mate-2 computer.

The eluants from the analytical column were monitored with the Waters 990 photodiode array detector at a fixed wavelength of 272 nm. The attenuator was set at 0.05 AUFS. The resulting chromatograms were recorded on a Waters 990 recorder and an NEC Power Mate-2 data system. Quantitation of HCTZ was based on the ratio of HCTZ peak area to that of the internal standard, HFTZ. Data presented in this investigation are mean ± SD unless otherwise indicated.

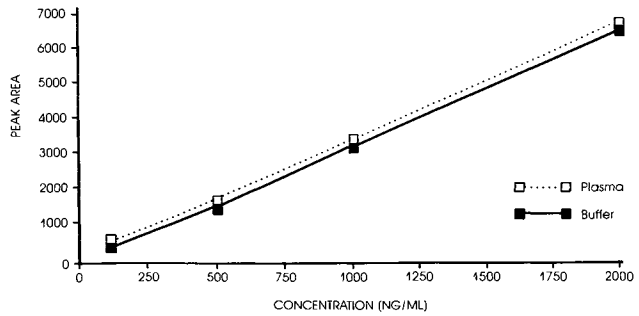


Fig. 4. Comparison of peak areas between HCTZ in rat plasma and HCTZ in 10 m*M* phosphate buffer, pH 7.2, under the same column-switching conditions. Each data point represents an average of four experiments.

RESULTS AND DISCUSSION

Specificity and Linearity

HCTZ in the plasma from three different species was well separated from the internal standard, HFTZ (Fig. 3), under the experimental conditions. Retention times observed from different occasions were 6.5 to 7.0 and 9.5 to 10.0 min for HCTZ and HFTZ, respectively. No interference in chromatogram was noted from the plasma constituents of the rats, dogs, or human (Fig. 3). Since HCTZ is excreted in the urine of rat or man almost completely as the intact sub-

Table I. Within-Run Precision and Accuracy of HCTZ Assay in Rat, Dog, and Human Plasma

Species	Predicted conc. (ng/ml)	Number of replicates	Observed conc. (ng/ml, mean ± SD)	CV (%)	Relative error (%)
Rat	2000	4	1986 ± 86	4.3	0.7
	500	4	503 ± 7.7	1.5	0.6
	100	4	98 ± 1.2	1.2	2.0
	50	4	52 ± 1.4	2.7	4.0
Dog	1000	4	1006 ± 34.6	3.4	0.6
	100	4	99.7 ± 3.8	3.8	0.3
Human	1000	4	1002 ± 7.0	0.7	0.2
	100	4	98.8 ± 2.1	2.1	1.2

stance (22,23), no interference from drug metabolite would be present.

The peak area ratio was linearly related to HCTZ concentration over a range of 25 to 2000 ng/ml for both rat and dog plasma and a range of 25 to 1000 ng/ml for human plasma, with a correlation coefficient of greater than 0.999 for all cases. Linear equations for the respective concentration range were $y = 0.000639x - 0.0115$, $y = 0.000725x - 0.0108$, and $y = 0.000789x - 0.0122$ for rat, dog, and human plasma, respectively. The quantitation limit of the assay for HCTZ in plasma could be as low as 25 ng/ml. Approximate signal-to-noise ratios at this concentration in rat, dog, and human plasma were estimated to be greater than 10. Good precision was also noted for this concentration as shown in Table II.

Relative Extraction Efficiency

The relative extraction efficiency of the extraction column was examined by comparing the peak areas of HCTZ and HFTZ in plasma to those in phosphate buffer, pH 7.2, under the same column-switching conditions. As shown in Fig. 4, the peak areas of HCTZ in rat plasma were very close to those of HCTZ in phosphate buffer at all concentrations tested (100 to 2000 ng/ml) and in both cases the slopes were found to be the same, suggesting that 100% of HCTZ in plasma might have been extracted and retained until elution. Observed in the same study using rat plasma, the peak areas of HFTZ in plasma and in buffer were 4009 ± 60 ($n = 16$) and 4890 ± 49 ($n = 16$), respectively, suggesting that 82% of HFTZ in plasma may have been captured by the extraction column. The plasma extraction ratios were not evaluated against the direct injection of the buffered solution onto the analytical column, because bypassing the extraction phase would create a different solvent flow pattern and different void volume, which might significantly affect the peak responses and thus introduce the artifact.

Reproducibility, Accuracy, and Stability

The within-run reproducibility and accuracy of the method were examined at several concentrations in plasma from three different species. These are shown in Table I. Over the range of 50 to 2000 ng/ml, the reproducibility of the assay was excellent, with coefficients of variation being in the range of 0.7 to 4.3%. Compared to the predicted concentrations, the observed concentrations of HCTZ also reflected the excellent accuracy of the method, with relative errors failing in the range of 0.2 to 4.0%. Between-run reproducibility was investigated using rat plasma as an example on three different occasions over a period of 2 months. A pool of rat plasma containing HCTZ at various concentrations (25 to 2000 ng/ml) was assayed on the day of preparation and on subsequent days. The plasma samples were stored at −20°C. As shown in Table II, the day-to-day precision and the accuracy were excellent.

The results of between-run reproducibility also suggest that HCTZ in frozen plasma was stable for at least 2 months. The question of stability can also be evaluated in another way. This was carried out using dog plasma as an example,

Table II. Between-Run Precision and Accuracy of HCTZ Assay in Rat Plasma on Three Different Occasions

Predicted conc. (ng/ml)	Observed conc. (ng/ml)					
	Occasion 1	Occasion 2	Occasion 3	Mean ± SD	CV (%)	Relative error (%)
2000	2005	1986	2041	2010.7 ± 27.9	1.4	0.5
1500	1502	1550	1452	1501.3 ± 49.0	3.3	0.09
1000	997	931	986	971.3 ± 35.4	3.6	2.9
500	503	503	499	501.7 ± 2.3	0.5	0.3
250	246	243	253	247.3 ± 5.1	2.1	1.1
100	96	98	96	96.7 ± 1.2	1.2	3.3
50	51	52	51.4	51.5 ± 0.5	1.0	3.0
25	24	29	25.7	26.2 ± 2.5	9.7	4.8

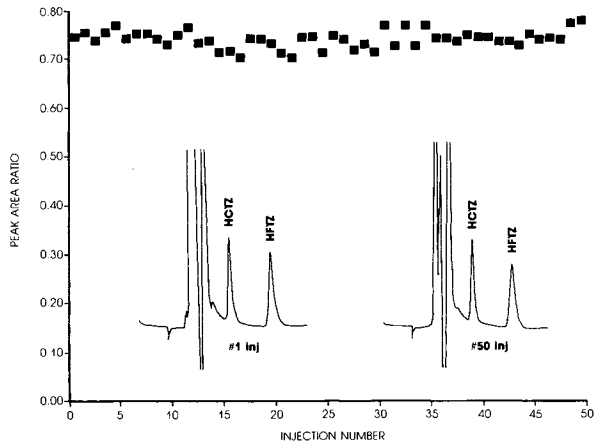


Fig. 5. Peak area ratios (■) of HCTZ (500 ng/ml) to HFTZ (750 ng/ml) in dog plasma obtained from 50 consecutive injections over a period of 20 hr and chromatograms of the first and the fiftieth injection.

where 50 consecutive plasma samples containing HCTZ and HFTZ were injected over a period of 20 hr at room temperature. As can be seen in Fig. 5, there were no dramatic changes in peak area ratio between the first and the last plasma samples. In fact, there was no significant change in chromatogram after 50 consecutive injections (Fig. 5), in terms of peak shape and retention time. These results thus suggest that at room temperature HCTZ and HFTZ in the buffered plasma samples were stable in the WISP autosampler overnight.

In Vivo Application

The present HPLC method has been applied to assay of dog plasma samples obtained from an oral dose range-finding study and to assay of rat plasma samples from a toxicology study. The results were then compared to those obtained from a modification of a previously published conventional HPLC method (13). The modification involved a cumbersome plasma extraction with hexane and methyl-t-butyl ether. As shown in dog plasma (Fig. 6) and in rat plasma samples (Table III), data obtained from the two different methods were comparable, suggesting that the present column-switching HPLC technique is a simple, reproducible, and rapid method for quantitation of HCTZ in plasma or other biological fluids.

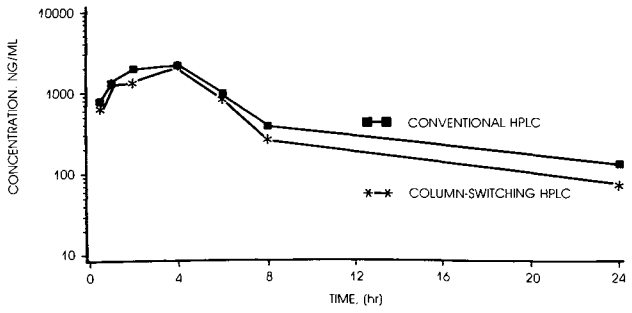


Fig. 6. Plasma concentrations in the dog receiving a dose of 10 mg/kg HCTZ determined by two different HPLC methods.

Table III. Plasma Concentrations (ng/ml) of HCTZ in Rat Plasma Determined with Two Different HPLC Methods; The Rats Were Given Doses of HCTZ Alone or in Combination with Diltiazem

Rat No.	Column-switching method	Conventional method
61[a]	1685.2	1581.5
62[a]	BQL[c]	BQL
63[a]	1905.8	1784.0
67[b]	159.0	200.2
68[b]	100.7	130.0
69[b]	248.6	212.3

[a] Rats daily received an oral dose of 40 mg/kg HCTZ and the blood sample was taken at Week 14, 1 hr after dosing.
[b] Rats daily received an oral dose of 10 mg/kg diltiazem and 2 mg/kg HCTZ and the blood sample was taken at the same schedule.
[c] Below quantitation limit.

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