Journal of Chromatography, 380 (1986) 450—454
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3164

Note

Determination of chlorothiazide in urine using reversed-phase high-performance liquid chromatography with ultraviolet detection

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(First received January 15th, 1986; revised manuscript received March 12th, 1986)

Chlorothiazide (CTZ), a benzothiadiazine diuretic (Fig. 1), was the first of numerous thiazide compounds known for their saluretic action. It has been used clinically in the treatment of hypertension, congestive heart failure, and other edematous conditions since 1957.

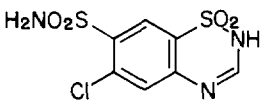

Fig. 1. Structural formula of chlorothiazide.

The traditional analytical procedure for CTZ in urine was a colorimetric method [1], in which base hydrolysis cleaved the heterocyclic ring to form the 1,3-disulfonamide. Subsequent diazotization and coupling formed a colored azo compound, which could then be quantified colorimetrically. These so-called Bratton and Marshall colorimetric assays [2, 3] exhibit high variability due to interferences from urinary constituents [4]. More recently, analysis of the drug in biological fluids has been performed either by liquid—liquid extraction, followed by high-performance liquid chromatographic (HPLC) analysis with UV detection [5—7], or by scintillation counting [8]. A reversed-phase HPLC method [9] has been developed in which no sample clean-up is required. However, no reproducibility data are provided and there is mention of some samples requiring ethyl acetate extraction to eliminate interference. The method described herein was developed to support a comparative bioavailability study. A ^{14}C-labeled intravenous dose was incorporated into the study to evaluate the HPLC assay by comparison with scintillation counting

0378-4347/86/$03.50

determination of CTZ. In light of the variability observed with previous methods [10] and the knowledge that CTZ is excreted unchanged in urine [11], it was desirable to develop a method for which the radioactive determinations were coincident with those obtained by HPLC. A reversed-phase HPLC method with UVdetection was developed, which requires no sample clean-up prior to injection into the HPLC system. The limit of reliable detection was 2 μg/ml, for which an intra-day coefficient of variation of 4.6% was observed.

EXPERIMENTAL

Materials and reagents

Methanol (HPLC grade), sodium perchlorate (HPLC grade), perchloric acid (reagent grade), anhydrous sodium carbonate (certified ACS), and sodium bicarbonate (certified ACS) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The water was purified by passing it through a Milli-Q filtration system (18 MΩ cm resistivity: cartridge sequence: Super-C, Ion-Ex, Ion-Ex, Organex-Q). The drug-free human urine was obtained from laboratory personnel. The CTZ analytical standard, 6-chloro-7-sulfamoyl-2*H*-1,2,4-benzothiadiazine-1,1-dioxide, was obtained from the sample respository Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.).

Assay procedure

Total urine collections were thawed to room temperature overnight. The specimens were stirred via magnetic stirring for 1 h prior to sampling. The stirring was maintained while taking the aliquot. Urine (1.0 ml) and methanol (0.4 ml) were transferred to a polypropylene centrifuge tube (1.5 ml). The tube was capped and the contents mixed by vortex and then centrifuged (5 min at 12 400 *g*). An aliquot (5 μl) of the supernatant was injected into the HPLC system.

Calculations

A standard curve of CTZ in urine was run daily with the clinical specimens. The calibration curve for urine was linear from 2 to 200 μg/ml. The equation for the resulting line was $y = 701x + 46.3$, where y is CTZ peak height and x is μg CTZ per ml of urine ($r^2 = 0.999426$). In addition, quality-control standards at 4.0 and 120.0 μg/ml of urine were assayed daily. The peak height of the drug from the unknown sample was employed to calculate its concentration from the standard curve.

Instrumentation

The HPLC system consisted of a Waters Model 720 system controller, two Waters Model 6000A pumps (one post-column), and a Waters Model 710B automatic sampler. Peak quantitation was performed by a Hewlett-Packard 3357 laboratory automation system. The detector was a Kratos Spectroflow 773 UV absorbance detector. The separation was provided by two analytical columns in series as follows: a Supelco LC-18 column (5 μm, 5 cm × 4.6 mm); a Waters μBondapak C_{18} column (10 μm, 30 cm × 3.9 mm); the guard column

was purchased from Bio-Rad (ODS, 10 μm, 4 cm × 4.6 mm). The 5-cm Supelco column was required to separate CTZ from the small peak at approximately 14.20 min as seen in Fig. 2. In the absence of an additional analytical column, this peak interfered with the accurate quantitation of low CTZ levels.

Instrumental conditions

The HPLC mobile phase was sodium perchlorate (0.1 *M*, adjusted to pH 4.0 with perchloric acid)—methanol (95:5). The mobile phase components were mixed and filtered under vacuum in a sonic bath. The flow-rate was 2.0 ml/min. The post-column pump was supplied with carbonate buffer, pH 10.0, which was degassed by sparging with helium. The post-column flow-rate was 0.5 ml/min. In order to obtain pulse-free solvent delivery from the post-column pump, several accumulator and restrictor coils, supplied by Waters Assoc., were placed in line with the high-pressure noise filter within the pump. The detector settings were as follows: wavelength monitored: 299 nm; absorption range: 0.005 a.u.f.s.; rise time: 2 s. The HP 3357 laboratory automation system provided peak quantitation with the following parameters: mV/min: 0.350; minimum area: 2500; report peak height (HEST, height external standard); retention time of 16.50 min (CTZ).

RESULTS AND DISCUSSION

The intra-day and inter-day precision and accuracy of the method are presented in Table I. Typical chromatograms are presented in Fig. 2.

The investigation, which led to the development of the described method, began with a liquid—liquid extraction sample clean-up followed by reversed-phase HPLC—UV analytical analysis similar to methods already mentioned. However, when comparisons were made between the amount of CTZ determined by the UV absorption of the chromatographic peak with that determined by scintillation counting of the corresponding eluent fraction, the

TABLE I

INTRA- AND INTER-DAY PRECISION AND ACCURACY OF THE METHOD

Nominal concentration (μg/ml)	Mean calculated concentration (μg/ml)	Coefficient of variation (%)
Intra-day (n = 6)		
2	1.94	4.6
4	4.18	1.9
8	8.20	2.1
20	20.43	2.0
40	39.42	2.0
80	79.86	1.8
120	117.11	2.4
200	195.52	2.0
Inter-day (n = 13)		
4.0	4.07	6.4
120.0	111.00	6.9

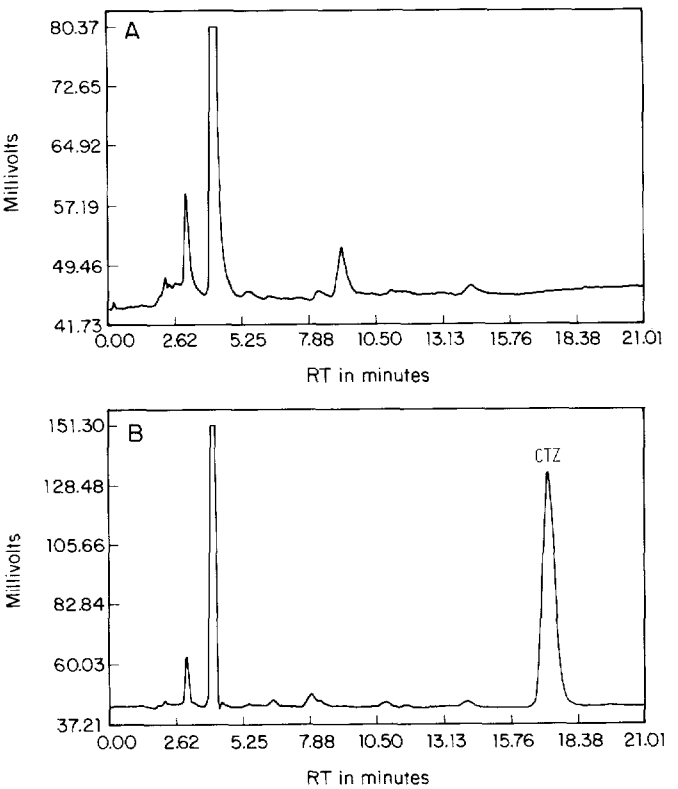

Fig. 2. Representative chromatograms of chlorothiazide (CTZ) in clinical subject urine. (A) Subject blank urine; (B) subject specimen post 125-mg intravenous dose (0—4 h, 136.83 μg/ml).

amount determined by the former method was as much as 25% lower. Therefore, doubt was cast upon the HPLC—UV method. After numerous experiments, it was found that several problems existed in the HPLC assay. An ethyl acetate extraction had been used for sample clean-up. In several clinical samples, we found the extraction efficiency of CTZ to be less than that in spiked urine samples. This observation was based on the comparison of the CTZ chromatographic peak height of a direct urine injection with that of an extracted sample. Several attempts were made to improve the extraction efficiency of clinical samples including pH adjustments, salt addition, and the application of heat to no avail. Since the extraction efficiency of the hydrolysis product (1,3-disulfonamide) was greater than that of CTZ in spiked urine samples, an attempt was made to base-hydrolyze the CTZ prior to extraction. Once again, coincidence of the HPLC and radioactive data was lacking. The structure of CTZ (Fig. 1), as well as the literature [12], suggest the possibility of the tautomeric forms shown in Fig. 3.

It was felt that some of the variability could be attributed to the chromatographic response of CTZ actually being two unresolved tautomers with different extinction coefficients. In fact, preliminary HPLC analytical column trials (utilizing different bonded phases) suggested the possibility of two

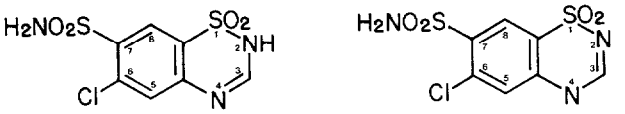

Fig. 3. Structural formulae of chlorothiazide tautomers.

partially resolved peaks. Analysis and comparison of the UV spectra of CTZ in base with that of the 2- and 4-methyl analogues of CTZ (i.e. no acidic proton) suggest that the anion resembles primarily that resonance structure in which the double bond resides in the 3—4-position [12]. The base form has a slightly higher extinction coefficient than the acid form. In addition, the base form exhibits a shift of the absorption maxima to higher wavelengths. As a result, an increase in UV absorbance was seen at the wavelength monitored in this HPLC assay. In order to obviate any variable ratios of the tautomers in the chromatographic peak of the clinical specimen versus that from a spiked urine sample, as well as enhance the sensitivity of the HPLC method, carbonate buffer, pH 10.0, was delivered post-column. With the HPLC conditions improved, the clinical specimens from the radiolabeled dose were injected directly into the HPLC system. On average, the HPLC data were found to be within 7% of that obtained via scintillation counting versus the aforementioned 25% discrepancy.

In addition, during the assay development, an apparent sampling problem was detected with clinical samples in that the results obtained for different aliquots from the same sample container did not concur. We found that reproducible aliquots could be obtained by stirring the entire clinical sample for a period of 1 h prior to sampling, as reflected in the assay procedure above. While this seems somewhat laborious and primitive, time limitations precluded the pursuit of a more elegant means of obtaining reproducible urine aliquots.

This present method has been employed for the routine analysis of 400 urine samples.

REFERENCES

1 J.E. Baer, H.L. Leidy, A.V. Brooks and K.H. Beyer, J. Pharmacol. Exp. Ther., 125 (1959) 295.
2 M.C. Meyer and A.B. Straughn, Curr. Ther. Res., 22 (1977) 573.
3 H. Sheppard, T.F. Mowles and A.J. Plummer, J. Pharm. Sci., 49 (1960) 722.
4 J.P. Hunt, V.P. Shah, V.K. Prasad and B.E. Cabana, Am. Pharm. Assoc. Acad. Pharm. Sci., 8 (1978) 195.
5 P.A. Tisdall, T.P. Moyer and J.P. Anhalt, Clin. Chem., 26 (1980) 702.
6 D.E. Resetarits and T.R. Bates, J. Pharmacokin. Biopharm., 7 (1979) 463.
7 E.T. Lin and L.Z. Benet, Am. Pharm. Assoc. Acad. Pharm. Sci., 8 (1978) 194.
8 J.H. Gustafson and L.Z. Benet, J. Pharmacokin. Biopharm., 2 (1974) 417.
9 V.P. Shah, V.K. Prasad, B.E. Cabana and P. Sojka, Curr. Ther. Res., 24 (1978) 366.
10 J.P. Hunt, V.P. Shah, V.K. Prasad and B.E. Cabana, Anal. Lett., 13 (1980) 135.
11 Merck Sharp & Dohme, Product Information Summary - DIURIL, August, 1973.
12 F.C. Novello, S.C. Bell, E.L.A. Abrams, C. Ziegler and J.M. Sprague, J. Org. Chem., 25 (1960) 970.