Use of Addition Calibration Technique for Determination of Acetaminophen and Hydrochlorothiazide in Human Urine by High-Performance Liquid Chromatography

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Abstract

The quantitation of target analytes in complex matrices such as biological samples requires special calibration approaches to compensate for additional capacity or activity in the matrix samples. A conventional calibration curve, obtained with standard solutions, is one of the most important calibration procedures for quantitation of target analytes in such matrices. However, these technique require a great number of reagents and material, and consume a considerable amount of time throughout the analysis. In this work, a new calibration procedure to analyze urine samples is proposed for the first time in chromatography procedures. The proposed calibration, called the addition calibration technique, was used for the determination of acetaminophen and hydrochlorothiazide in urine samples. The results obtained for the proposed calibration mode were compared to those obtained using standard addition and standard calibration techniques. The proposed addition calibration was validated by statistical studies between results obtained by the addition technique and conventional techniques, using the ANOVA test and linear regression. The results demonstrated good agreement among them. The performance of the analytical method was evaluated. Relative standard deviation, limit of detection, and limit of quantification are respectively 0.5–0.6%, 0.169–0.75 µg/mL, and 0.565–2.5 µg/mL. Linear range falls within the range of 0.3 to 63.8 µg/mL for both compounds. Accuracy ranged between 94% and 101%.

Introduction

The analgesic acetaminophen (*N*-acethyl-p-aminophenol) and the diuretic hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide) are drugs extensively recommended by health care professionals and are widely consumed by the population. The former is used to reduce pain without causing numbness or loss of consciousness (1). The second is used for the treatment of high blood pressure, edematous conditions, and many forms of renal disorders (1–3). These compounds can cause many adverse effects in healthy humans. The excessive consumption of analgesics causes serious intoxication problems that can result in severe damage to the kidneys and liver (1,2,4,5). An overdose of thiazide diuretics or a long period of therapy can cause metabolic disorders such as hypoglycemia and glycosuria, and they are suspected of being associated with the increase of carcinoma risk factors in renal cells and cervical cancer (2). Because diuretics reduce the body mass of athletes and lower the urine concentration of other doping agents, these compounds have been banned in sports events since the 1988 Olympic games (6).

A number of analytical methods reported in the literature for the determination of acetaminophen (AC) and hydrochlorothiazide (HCT) in several types of matrices, individually or in combination with other drugs, as well as their main metabolites were developed. AC has been determined by colorimetric, spectrophotometric, or chromatographic methods (4,5,7–9). HCT has been determined by voltammetric, capillary electrophoretic, spectrophotometric, or chromatographic methods (10–20). Recently, high-performance liquid chromatography (HPLC) equipped with an UV or electrochemical detector has been used with more frequency because it increases the sensitivity and selectivity of the analysis (9).

However, regardless of the instrument or technique choice, an adequate calibration technique is fundamental to perform the correct determination of drugs in complex matrices because errors in the analytical signal can occur because of matrix interference. Among the calibration techniques frequently used to determine drugs in biological matrices is the standard calibration technique using a drug-free biological matrix (6,7) or the HPLC mobile phase (5,8,14,17,18) like solvent, standard addition calibration, and internal calibration (4). In spite of the standard addition calibration taking the matrix effects into consideration, it not only requires a great number of reagents and material, but also consumes a considerable amount of time throughout the analysis. So, the standard addition calibration procedure is not often used for drug quantitation in biological matrices.

Concerning the error produced by conventional calibration methods, some authors (21–23) have proposed a new calibration technique, called addition calibration technique, to compensate for additional interference of the matrix samples. The addition calibration technique uses the slope obtained in the standard addition calibration curve (spiking the sample matrix with the analyte before build the calibration curve) for quantitation of the analyte in the sample matrix. The aforementioned calibration

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technique was used for quantitation of lead in blood using electrothermal atomic absorption spectrometry (ETAAS) (21), arsenic in sediments, coal, and fly ash using ETAAS (22) and zinc in biological samples by flame atomic absorption spectrometry (FAAS) (23).

This study is the first time that the addition calibration technique has been applied to the quantitation of AC and HCT in human urine. The results obtained from the proposed calibration technique were statistically compared to those obtained from conventional calibration techniques.

Experimental

Apparatus

All analyses were carried out with an HPLC system from Varian. The chromatography system consisted of two POSTAR 210/215 pumps, a POSTAR 410 auto-sampler with capacity for 84 vials, and 100-µL loop and a POSTAR 320 UV–vis detector. The peak areas were integrated automatically by computer using the Varian Star Chromatography Workstation version 5.0 software program.

Chemicals

Acetaminophen (99.61%) and hydrochlorothiazide (100.20%) were kindly supplied by Instituto Nacional de Controle de Qualidade da Saúde – Fundação Osvaldo Cruz. Methanol and acetonitrile (Merck, Darmstadt, Germany), sodium dihydrogen phosphate (Vetec, Rio de Janeiro, Brazil) and phosphoric acid (Biotec) used in this work were of analytical grade and were used without further purification. Deionized water was obtained in a Milli-Q purification system (Millipore) and used to prepare solutions.

Procedure for HPLC

Chromatography conditions

Solutions and mobile phases were prepared at the time of use. For the determination of AC the mobile phase water–methanol (85:15 v/v) and MICROSORB-MV 100 C18 (5 µm, 250 × 4.6 mm) analytical column were used. The mobile phase flow rate was 1.2 mL/min, and the detector was set at 243 nm. For the determination of HCT the mobile phase 0.1M sodium dihydrogen phosphate–acetonitrile (84.5:14.5 v/v, pH 3.0) and CHROMPACK Inertesil–ODS C18 (5 µm, 250 × 4.6 mm) analytical column were used. The mobile phase flow rate was 2.0 mL/min and the detector was set at 254 nm. In all analyses 10 µL of solution was injected and separation occurred at room temperature.

Standards

Individual stock solutions were prepared by dissolving the appropriate amount of AC in water–methanol (75:25) and HCT in methanol–water (70:30), respectively, to yield a final concentration of 300 mg/mL. Work solutions for each drug were obtained by appropriate dilutions of stock solutions with the respective mobile phase and drug-free urine.

Preparation of urine sample

People received a single doses of AC (750 mg) or HCT (50 mg) were the volunteers. A total of 10 urine samples containing AC and HCT were collected within intervals of 1–8 h and 3–9 h after administration of AC and HCT, respectively. The samples were stored in a freezer at –20°C until the analysis procedure.

The procedure for sample pre-treatment was the same as suggested by Farthing et al. (14). All 10 samples collected from the individuals administered AC were prepared by adding 200 mL of urine and 1800 mL of water into a glass vial, and agitating in a vortex for 10 s. The diluted urine was filtered, and 10 µL of the filtrate was injected into the HPLC system. The urine samples collected from individuals that took HCT were prepared by adding 400 mL of urine and 1800 mL of water into a glass-vial, and agitating in a vortex for 10 s. The diluted urine was filtered and 10 µL of the filtrate was injected into the HPLC system.

Calibration procedures

In this study, standard calibration using a drug-free matrix or mobile phase solvent, standard addition, and the proposed addi-

Table I. Analytical Parameters of Merit Obtained Through Standard Calibration Curves for the Drugs AC and HCT

Compounds	Matrix	Range (µg/mL)	LOD* (µg/mL)	LOQ† (µg/mL)	Equation	r‡
AC	Mobile phase	0.024–24	0.071	0.235	$y = 21326x + 146.66$	0.9998
	Drug-free urine	0.3–24	0.169	0.565	$y = 22124.7x + 1232$	0.9999
HCT	Mobile phase	0.06–24	0.166	0.555	$y = 29210x + 361.94$	0.9995
	Drug-free urine	0.6–63.77	0.75	2.5	$y = 16812x + 2845$	0.9997

* LOD = limit of detection; † = LOQ limit of quantification; ‡ r = correlation coefficient.

Table II. Comparative Study Among the Three Calibration Methods for Determination of AC in Urine

	Calibration Methods					
	Standard		Addition		Analyte addition	
Samples	(µg/mL)	± S	(µg/mL)	± S	(µg/mL)	± S
1	1.02	0.05	1.02	0.05	1.02	0.06
2	1.46	0.07	1.38	0.09	1.14	0.21
3	2.82	0.00	2.64	0.00	2.67	0.02
4	13.85	0.26	13.67	0.44	13.83	0.20
5	6.07	0.13	5.89	0.07	5.9	0.25
6	2.75	0.04	2.6	0.01	2.33	0.03
7	8.07	0.04	7.65	0.04	7.75	0.19
8	1.58	0.07	1.46	0.07	1.48	0.18
9	2.93	0.04	2.75	0.04	2.49	0.39
10	15.47	0.00	14.74	0.00	14.61	0.17

tion calibration procedure were compared for the determination of AC and HCT in urine samples.

Standard calibration was carried out using drug-free urine or mobile phase to obtain the working standard solutions in range of 0.024–24 µg/mL and 0.06–64 µg/mL for AC and HCT, respectively.

In the calibration performed by means of the standard addition technique, 1.0-mL aliquots of the diluted urine samples were transferred to volumetric flasks, to which different amounts of the 300 µg/mL AC or HCT stock solution were added. Then, a total of 10 calibration curves for each drug were necessary to determine their concentration in these samples. The addition calibration technique, which is the goal of this study, is a simplification of the standard addition technique. In this case, the slopes obtained in the standard addition calibration curves from urine samples were used, respectively, for quantitation of AC and HCT in urine matrix. In this sense, a standard addition curve for each drug was turned into a standard curve (here, called addition calibration) and this new curve was used to determine the drug concentration for all other samples (21–23).

The samples were analyzed, in triplicates, for each calibration technique used here.

Statistical methods

Each sample was analyzed three times by standard calibration, standard addition calibration, and addition calibration. The results are reported as means and standard deviations (S.D.). Differences between the addition calibration and the other calibration techniques were tested using the ANOVA test. The level of statistical significance was at 5%. Because of the wide range of AC or HCT concentration found in urine samples, the calibration techniques were also compared by linear regression for the whole set of values ($n = 10$).

Results and Discussion

To achieve the goal of this work the following sequence consisted of three steps: (*i*) the choice of a simple chromatographic procedure was focused on to determine AC and HCT, separately. The procedure choice should not contain tedious steps such as extractions and clean up procedures and should allow us to obtain good efficiency, selectivity, and sensibility for the target analytes. In this sense, the procedure suggested by Farthing et al (15) was adapted in our laboratory. (*ii*) Three different calibration techniques were used to determine AC and HCT concentration in urine samples. (*iii*) The question of whether there were significant differences among the three calibration techniques used to determine AC and HCT concentration was investigated. In this study, statistical methods were used.

Table III. Comparative Study Among the Three Calibration Methods for Determination of HCT in urine

	Calibration Methods					
	Standard		Addition		Analyte addition	
Samples	(µg/mL)	± S	(µg/mL)	± S	(µg/mL)	± S
1	1.25	0.06	1.44	0.06	1.81	0.11
2	1.98	0.05	2.17	0.05	2.26	0.05
3	1.81	0.03	1.99	0.03	2.09	0.23
4	4.98	0.05	5.21	0.05	6.02	0.01
5	3.21	0.15	3.42	0.16	4.15	0.21
6	4.46	0.12	4.9	0.12	4.73	0.11
7	2.29	0.00	2.47	0.00	2.35	0.08
8	2.43	0.05	2.58	0.01	2.65	0.03
9	2.89	0.04	3.01	0.12	2.77	0.02
10	14.5	0.1	14.82	0.10	14.91	0.15

Table IV. ANOVA Test for the Results Obtained from AC and HCT in Urine Samples Using Three Quantitative Techniques

Source of variation	Sum of square		Degrees of freedom		Mean squares		F-test ($p = 0.05$) F* = 3.35	
	AC†	HCT‡	AC	HCT	AC	HCT	AC	HCT
Between groups	0.4368	0.780	2	2	0.2184	0.390	0.0081	0.0255
Within groups	722.97	413.45	27	27	26.776	15.313		
Total	723.41	414.23	29	29				

* F = F tabulated; †AC = acetaminophen; ‡HCT = hydrochlorothiazide.

Analytical parameters of merit

The methods adapted to determine AC and HCT were validated in regards to linearity, limit of detection (LOD) and quantitation (LOQ), precision, accuracy, and specificity. Table I contains information about these parameters when using mobile phase or drug-free urine as solvent in the working solution preparation. Peak areas of AC and HCT in calibration standards were proportional to their concentrations in drug-free urine over the range tested 0.3–24 µg/mL and 0.6–63 µg/mL, respectively. The calibration curves were fitted by linear least-square regression and showed coefficients of determination greater than 0.9995. The chromatographic separation achieved to determine AC and HCT showed good selectivity and shapes of chromatographic peaks in spite of the procedures including no clean up steps. The specificity, in relation to other urine components, can be observed in Figure 1 (1A for AC and 2B for HCT). The LOD and LOQ were defined as the concentration of an analyte that gives an equivalent response to three and ten times the standard deviation (SD) of the blank divided by the slope of the correspondent calibration curve, respectively. The precision of the sample preparation method in terms of peak area and retention times for both analytes was obtained through the calculation of

relative standard deviation (RSD) of three analyses of the same sample and they were better than, respectively, 1.6% and 0.5% for both analytes. The accuracy of the method was obtained by spiking some naturally contaminated samples with the studied drugs and determining their recovery. The values obtained were between 94–101% for both analytes (n = 5).

Comparison of calibration procedures

The sequence of this study was based on the comparison of urine concentration of AC (or HCT) obtained by standard calibration using drug-free urine, standard addition calibration, and addition calibration. For both urine samples containing AC and HCT the slopes of the calibration curves resulted from the standard addition technique were approximately equal, indicating the presence of similar matrices. The similarity among the urine matrices indicates that these samples contain the same interferences acting in the same way on the target analyte. From the results of the standard addition calibration and the addition calibration, the calibration curves determining the concentration of AC (or HCT) in urine could be determined. Thus one standard addition calibration curve was chosen to generate the addition calibration curve for AC and another for HCT. In this study, for both analytes, the standard addition calibration curve chosen was the one that was the closest to the average angular coefficient slope calculated from the standard addition curves. To generate the new curve (addition calibration) the value of AC (or HCT) concentration (previously determined in the urine sample using the selected standard addition curve) was added to the original amount of AC (or HCT) via stock solution, and the signal was the same as that obtained from the standard addition curve, generating a new calibration curve (addition calibration). As an example, the urine sample chosen to generate the addition calibration curve for determination of AC was spiked with AC standard solution to give an AC final concentration of 0, 0.5, 0.9, 24, and 50.07 µg/mL. The chromatographic area obtained for these solutions were, respectively, 32667, 48566, 53843, 640160, and 1180078. The correspondent equation and AC concentration were y = 23172x + 41628.9 and 1.8 µg/mL, respectively. The value of the AC concentration was added to that obtained for spiked with AC standard solution. So, the new values of AC concentration were 1.8 µg/mL (32667); 2.3 µg/mL (48566); 2.7 µg/mL (53843); 25.8 µg/mL (640160), and 51.87 µg/mL (1180078). The correspondent chromatographic areas were not changed and they are indicated in brackets. The new equation obtained for this data was y = 23142x + 100. This new curve was used to obtain AC concentration on the 10 urine samples and was also used to make comparisons among the techniques of standard addition and addition calibration. The same procedure was carried out for determination of HCT.

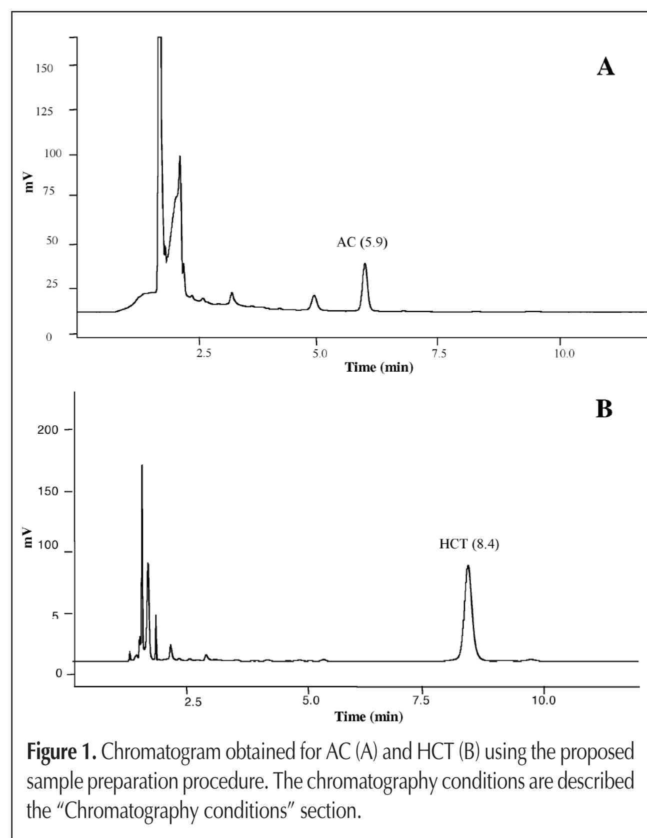

Figure 1. Chromatogram obtained for AC (A) and HCT (B) using the proposed sample preparation procedure. The chromatography conditions are described the "Chromatography conditions" section.

Data on AC and HCT content in urine samples expressed as means and standard deviation referring to calibration techniques are shown in Table II and III, respectively. The proposed calibration technique gave comparable results in relation to the conventional techniques, and we did not find any difference (P > 0.05) between AC (or HCT) concentrations after applying a paired two-tailed Student's t-test. The ANOVA test was also applied to each drug in urine samples. F-values were computed and compared to the standard tabulated value using a significant level of P = 0.05. From the standard table, for n_1 = 2 and n_g = 27 (P = 0.005), the value of F is given as 3.35. The calculated F-values did not exceed the tabulated values of F in the analysis of variance, indicating that there was no significant difference among the calibration methods. ANOVA's results were illustrated in Table IV. Because of the wide range of AC and HCT concentration in urine samples, it was possible to compare the results obtained by the addition technique and the conven-

Table V. Relevant Statistical Parameter of the Linear Regression Between the Results Obtained from the Calibration Techniques in Naturally Contaminated Urine Samples

Concentration range (µg/mL)	Number of points	Correlation coefficient (r)	Slope, (b ± tSb*)	Intercept (a ± tSa†)
AC	Addition vs	Analyte addition	calibration	
1.02–13.85	10	0.996	0.99 ± 0.01	0.12 ± 0.07
	Addition vs	standard	calibration	
1.02–13.85	10	0.999	0.97 ± 0.01	–0.05 ± 0.00
HCT	Addition vs	Analyte addition	calibration	
1.25–4.98	10	0.996	0.99 ± 0.03	–0.13 ± 0.18
	Addition vs	standard	calibration	
1.25–4.98	10	0.999	1.01 ± 0.01	0.17 ± 0.04

* 95% confidence limits for slope; † 95% confidence limits for intercept.

tional techniques by linear regression for the whole set of values. The results of these comparisons with the correspondent values for the correlation coefficient, slope and intercept values are presented in Table V. The value of the slope is very close to the theoretical value of unity and the value of intercept does not differ significantly from zero for all comparisons, indicating no difference among AC (or HCT) concentration obtained by the calibration techniques. In this sense, it is possible to generate just one curve using the sample matrix to quantify the other samples. In this work, it was possible to generate one calibration curve to obtain the AC concentration of the 10 samples. The same procedure (i.e., generates just one calibration curve for HCT in the urine matrix and used it for obtaining the HCT concentration from the 10 samples).

Conclusions

The addition calibration technique is applicable for the determination of AC and HCT in urine samples. The addition calibration proved to be reliable for both spiked and natural samples (i.e., volunteers administered AC or HCT), in a wide range of drug concentrations, when plotted against conventional calibration techniques. In addition, the AC and HCT concentration obtained by addition calibration does not present differences from other calibration techniques when tested with a paired two-tailed *t*-Student test and the ANOVA test. The proposed calibration requires fewer reagents and less material than standard addition calibration, it is simpler and less time consuming.

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