Quantitative Analysis of Hydrocortisone in Human Urine Using a High-Performance Liquid Chromatographic–Tandem Mass Spectrometric–Atmospheric-Pressure Chemical Ionization Method

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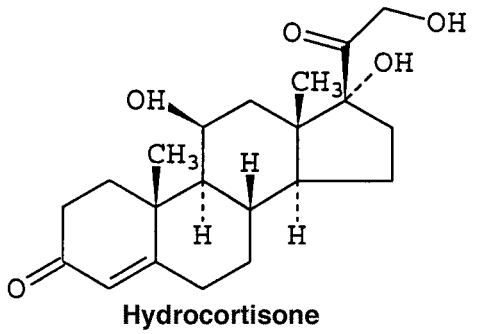
Abstract

In this study, the development and validation of a method of analysis for 11,17,21,-trihydroxypregn-4-ene-3,20-dione (hydrocortisone, cortisol, HC) using high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS) with atmospheric-pressure chemical ionization (APCI) is reported. This is the first report of the systematic development and validation of an HPLC–MS–MS method for the quantitation of HC in synthetic human urine with a deuterated internal standard. Prior to LC–MS–MS analysis, the only sample preparation used was the dilute-and-shoot technique prior to LC–MS–MS analysis. In this study, an analysis time of less than 3 min is achieved. The results show freedom of interference from other analytes such as analogous steroids. Validation parameters such as specificity/selectivity, limit of quantitation (LOQ), linearity, precision, accuracy, ruggedness, stability, and system suitability are evaluated for this method. The LOQ is 5 ng/mL with an 8% relative standard deviation (RSD). For calibration standard curves, an average linear response for a 3-day validation (R^2 = 0.997) over the range of 5 to 500 ng/mL is obtained. The interday precision %RSDs are 7.2, 5.0, and 5.2 for 15, 75, and 300 ng/mL, respectively. Also, brief comparisons of the dilute-and-shoot and liquid–liquid extraction techniques for this analyte are discussed.

Introduction

It has been found that in the human body increased levels of total body cortisol (11,17,21,-trihydroxypregn-4-ene-3,20-dione; hydrocortisone; HC) are associated with depression. The level of HC is generally higher in women and increases in all humans with advancing age. The immunosuppressive effects of HC cause it to act as one of the primary mediators of stress response. This factor makes it useful as a biochemical marker for antidepressant drugs. HC is interconverted by tissue-specific enzyme systems in many organisms (1). The extent of conversion may vary from individual to individual or from one time to another. The structures of HC and its deuterated internal standard (IS) are shown in Figure 1.

The increase of HC levels in urine, plasma, and saliva has been measured as a human response to stress for many years. The assay of single blood or saliva samples, however, is inherently imprecise because of the sporadic nature of HC secretions in humans as well as the difficulty in obtaining adequate sample sizes. Another



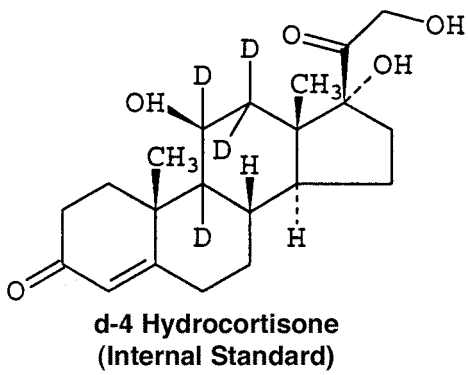


Figure 1. Chemical structures of HC and the IS.

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problem with analyzing blood or saliva samples is that the resulting measurements are of total HC levels (including protein-bound) as opposed to the free hormone, which is generally a much smaller proportion. Urine is composed of waste products from blood that collect in the kidney and are eliminated from the body through the urinary system. It is more than 95% water by weight with urea, sodium, chloride, potassium, creatinine, uric acid, and ammonia being the principal solutes. For the purpose of biochemical analysis, urine has several important advantages over plasma or saliva. It is more stable, and a typical sample size is larger and more easily obtained, particularly for children and the elderly. Also, the measurement of HC in urine has been shown to be a reliable index of total HC in plasma (2).

Although several methods for measuring HC in plasma, urine, and saliva have been reported (3–11), there are inherent limitations with most of them—lack of specificity, longer assay times, and interferences from naturally occurring components in the matrices. The method most commonly used for the analysis of HC is radioimmunoassay. However, because it measures the level of total HC in the matrix, it can overestimate the level of free HC. When reviewing previous studies, it becomes apparent that a fast, sensitive, and selective bioassay method for the quantitation of HC levels in biological matrices would be highly beneficial.

In this study, we report a simple and sensitive method using liquid chromatography (LC)–atmospheric-pressure chemical ionization (APCI)–tandem mass spectrometry (MS) for the quantitation of HC in human urine. The validation of this method was performed using synthetic human urine (12,13) with deuterated HC as the IS. The only sample preparation involved prior to LC–MS was a simple dilution with water. An analysis time of less than 3 min was achieved. The results showed freedom of interference from other analytes such as analogous steroids. This study shows that the method performed well in terms of our validation criteria of sample solution stability, linear response over a wide range of concentrations, high precision, and accuracy.

Experimental

Apparatus

Experiments were performed on a Hewlett Packard 1090 Series II LC using an Eclipse XDB-C18 analytical column (4.6 × 75 mm, 3.5 µm), a Zorbax XDB-C18 guard column (4.6 × 12.5 mm, 5 µm) (Mac-Mod Analytical, Chadds Ford, PA), and Rep Frit (TAN) A-101X 2-µm stainless steel frits (0.062-inch disc diameter and thickness, 0.25-inch o.d.) (Upchurch Scientific, Oak Harbor, WA). MS in positive-ion mode was done on a Finnigan TSQ 7000 with a tandem quadrupole MS (ThermoQuest, San Jose, CA) using a Finnigan electrospray ion source, an APCI interface at a flow rate of 1.0 mL/min, and a Digital UNIX version 3.2 computer operating system (Digital Electronic Corporation, Nashua, NH). Nitrogen (grade 5.0) was used as the nebulizing gas, and the vaporizer was maintained at a temperature of 500°C.

Reagents and procedures

HC was obtained from Aldrich and its deuterated IS (HC-d4) from Cambridge Isotope Laboratories. Trifluoroacetic acid and methanol were obtained from Fisher. The isocratic system for HPLC consisted of solvent A containing 0.003% TFA–water (40%) and solvent B containing 0.003% TFA–methanol (60%) at a flow rate of 1 mL/min. The column temperature was 45°C and the injection volume 50 µL. The following conditions were used for MS: the ionization mode was APCI in positive ion with a capillary temperature of 200°C, a vaporizer temperature of 500°C, and a manifold temperature of 70°C; the electron multiplier was set at 2700 V; the high energy dynode was 15 kV; the sheath gas was nitrogen at 80 psi; the auxiliary gas was turned off; the corona current was 5.00 µA; the collision cell pressure (argon) was approximately 2.2 mtorr, a selective reaction monitoring (SRM) mode was used; and the analysis time was 3.0 min.

Analytical standards and quality-control samples

Analytical standards and quality-control samples (QCs) were prepared from independent weighings and by the addition of known quantities of standard solutions in order to control synthetic blank urine (500 µL). The concentrations of the analytes were 5, 10, 20, 50, 100, 200, 400, and 500 ng/mL. The QCs were prepared at low (15), mid (75), and high (300) nanograms per milliliter and were processed in duplicate.

Sample preparation

Five-hundred-microliter samples of urine and QCs were thawed at room temperature for approximately 90 min. The IS, 25 µL of the 350-ng/mL working standard, and 500 µL of water were added to all the vials, which were then vortexed for 30 s and injected into the LC–MS.

Method validation

Method validation consisted of demonstrating or establishing method specificity, LOQ, linearity, precision, accuracy, stability, and ruggedness. All of the method-validation experiments were carried out using synthetic urine. The approach used to address each validation parameter is briefly described as follows:

Specificity/selectivity

Specificity or selectivity are measures of the degree of interference in the analysis of complex sample mixtures. Specifically in this study, it is the freedom of interferences present in the blank samples. Six different batches of human urine were analyzed using the normal procedure to check for the interference of other analytes.

LOQ

LOQ is defined here as the analyte level that gave a signal-to-noise ratio greater than 3.

Linearity

Linearity was determined by analyzing standard curves at ten different concentrations over the range of 5.0 to 500 ng/mL.

Precision

Three levels of calibration standards were repeated six times a day for three days in order to assess interassay precision. The six replicates on each day were further used as separate data sets for intra-assay precision.

Accuracy

In order to demonstrate the recovery percentage of the analyte upon the preparation from synthetic urine, the following procedure was used. Three low (QL) and three high (QH) QCs were prepared using the normal procedure described previously. Neat solutions at QL and QH levels were prepared by transferring 37.5 µL of 200-ng/mL (QL) or 4000-ng/mL (QH) analyte QC working solution, 25 µL of 350-ng/mL internal spiking solution, and 0.9625 mL of water into an HPLC vial and mixing well.

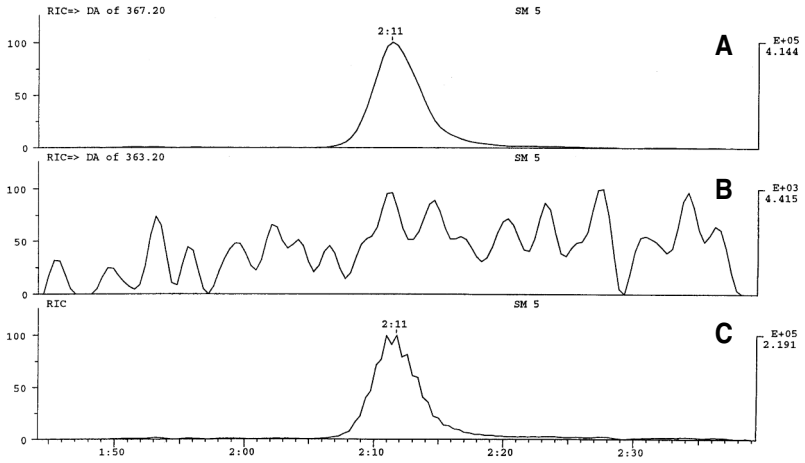

Figure 2. LC–MS–MS multiple-reaction monitoring ion current chromatograms of 5 ng/mL HC in diluted synthetic urine (LOQ) obtained by SRM of (A) *m/z* 367.2 to 121.2 for the IS, (B) *m/z* 363.2 to 121.2 for HC, and (C) relative ion chromatogram.

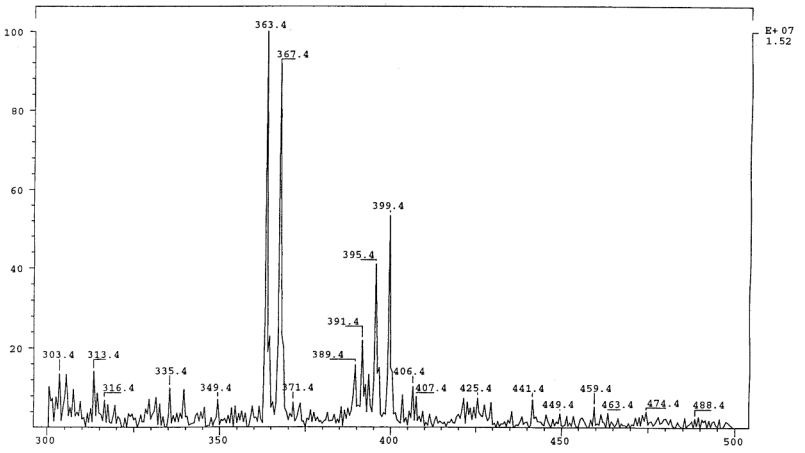

Figure 3. MS positive-ion APCI mass spectra of HC and the IS (the parent ion mass spectra of HC and the IS).

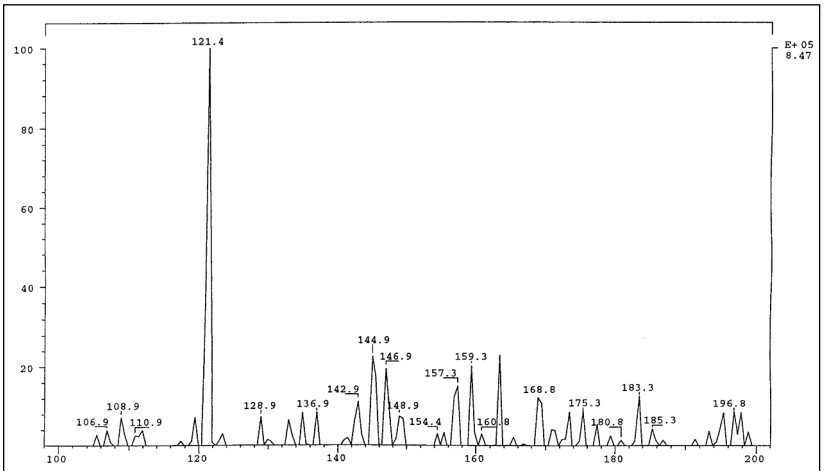

Figure 4. Product ion spectrum of HC.

Stability

The stability of the analyte in synthetic urine upon freeze–thaw cycles was demonstrated using three sets of QCs (A, B, and C) containing three each of low, medium, and high QCs. For the first cycle, set A QCs were frozen at –70°C for 30 min and stored at –15°C until analyzed. The samples were thawed for 2 h at room temperature and analyzed. For the second cycle, set B QCs were frozen at –70°C for 30 min, thawed at room temperature for 2 h, refrozen at –70°C for 30 min, and stored at –15°C until analyzed. The samples were thawed for 2 h at room temperature and analyzed. In the third cycle, set C QCs were again frozen at –70°C for 30 min, thawed at room temperature for 2 h, refrozen at –70°C for 30 min, thawed at room temperature for 2 h, refrozen at –70°C for 30 min, and stored at –15°C until analyzed. The samples were thawed for 2 h at room temperature and analyzed.

In order to demonstrate the bench stability of the extracted samples, twelve samples containing all analytes of interest were prepared using the normal procedure. The samples were analyzed as follows: three at the beginning of the first day of the validation, three at the end of the first day, three during the second day, and three during the third day. For the preparation of benchtop stability test samples, 6 mL of blank synthetic urine was pipetted into a 50-mL beaker. Then, 150 µL of the urine was removed, discarded, and replaced with 150 µL of the 4000-ng/mL analyte spiking solution. This was mixed with a stirring bar for 15 min. Then, 300 µL of the IS spiking solution was added to the beaker and mixed for 30 min. After this, 1.0 mL of the solution was transferred into each of the twelve HPLC vials.

Results and Discussion

Method development for HC in human urine

One of the driving forces for this study was to establish a simple, fast, accurate, and sensitive LC–MS–MS procedure for the determination of HC levels in human urine. The HC concentration in urine typically ranges from 20 to 200 ng/mL (4,5). When developing and validating methods for assaying HC in urine, the urine must be stripped of HC. The stripping process can be time consuming

and expensive and not always completely effective. The stripped biomatrix in some circumstances is not a reasonable facsimile of the biomatrix, which leads to inconsistencies in assay behavior. An easier, cheaper, and faster approach is to use synthetic human urine.

For our method development and validation, we used synthetic urine, which has all the characteristics of urine such as osmolality, specific gravity, and pH as well as the consistent presentation of human-urine biomatrix (12,13). This factor greatly improves the reliability of assay behavior. Another advantage to this method is that sample preparation is simple, requiring only the dilution of the sample with water prior to LC–MS, thus allowing for the preparation and analysis of a minimum of 20 samples per hour. The use of LC–MS–MS for HC bioassay improves the lower LOQ compared with previous methods using LC with UV detection (11).

Figure 2 shows the LC–MS–MS chromatograms of 5 ng/mL diluted synthetic human urine. These chromatograms clearly indicate the lack of interference when using diluted samples for LC–MS analyses as well as the improvement in detection limits. These advantages (along with the simplicity of this method) would be of great benefit in many other LC–MS–MS applications. Figure 3 presents MS positive-ion APCI mass spectra of the parent ions of HC and the IS. APCI was carried out in positive-ionization mode using nitrogen as the sheath gas at 80 psi. A full-scan mass spectrum of HC in Q1 mode showed an intense protonated molecular ion $[M+H]^+$ that appeared at *m/z* 363 for HC and *m/z* 367 for IS. Figure 4 represents the MS–MS experiment using a collision offset energy of 27 eV for HC and IS and a collision cell pressure of 2.1 mtorr that produced fragmentation of the $[M+H]^+$ ions in the collision cell (Q2). The $[M+H]^+$ ions of both analytes produced daughter ions at *m/z* 121.

Method validation

Method validation in this work consisted of demonstrating or establishing method specificity, LOQ, linearity, precision, system repeatability, accuracy, ruggedness, solution stability, and system suitability. The measurement of endogenous HC in urine samples would require the use of steroid-stripped urine for the preparation of standard curves and QCs. Because this steroid-stripping process is laborious and expensive, we developed an alternative technique using a synthetic urine with an analogous compound (HC-d4) for the purpose of calibration, which was used for all of the method validation experiments. The approach used to address each method validation parameter is described in the following sections:

Specificity/selectivity

Selectivity is a measurement of the degree of interference in the analysis of complex sample mixtures. Specifically in this study, it is the freedom from analyte metabolites or other interferences present in the blank samples. The chromatograms in Figure 5 shows the absence of interference from degradation products or metabolites, which was crucial to the targeted analysis of our analyte. A test was made of possible interferences from analogous steroids including cortisone; α-cortolone (5β-pregnene-3α, 17α,20α,21-tetrol-11one); β-cortolone (5β-pregnene-3α,17α,20α,21-tetrol-11one); 5α-pregnane-3α,11β,17α,21-tetrol-20dione; tetrahydrocortisone(5β-pregnane-3α,17α,21-triol-11-20dione); fluticasone propionate; flunisolide; β-OH flunisolide; and prednosone. These steroids did not produce any potential interferences in this bioassay. Also, we spiked the blank urine with only the IS in order to check for any interference from the IS with HC, and (as shown in Figure 2) there was none. These results clearly indicated the freedom of interference from any other analytes. The assay was considered adequately selective when no endogenous human-urine components (at the same *m/z* of the analytes of interest) eluted at the same retention times of the analytes of interest.

LOQ

For the HC in urine, the LOQ was defined as the analyte level that produced a signal-to-noise level ratio greater than 3 with the acceptance criteria for the accuracy and precision of replicate standards set at 20% or less of the theoretical value. The LOQ in urine (as determined by the intrarun accuracy and precision of replicate standards) was 5 ng/mL for HC. Figure 2 shows that the LOQ for HC was 5 ng/mL with a signal-to-noise ratio of 5. HC LOQ samples had an acceptable intrarun precision of 8.8%.

Linearity

Calibration curves were generated by a least-squares linear-regression analysis using the peak-height ratio of the analyte to the IS. Linearity was determined by analyzing standards at ten different concentrations over the range of 5 to 500 ng/mL. Figure 5 is a graph of back-calculated concentrations of the standard curves for HC during the three-day validation, which consisted of two standard curves each day for three days. The relative standard deviation (RSD) percentage of the interday results were determined by pooling the individual assay results of the calibration standards over three separate days. For all six curves, the average correlation coefficient for the linear best fit was 0.998. The %RSD for the slope and the coefficient correlation for all six standard curves were 3.26 and 0.04, respectively.

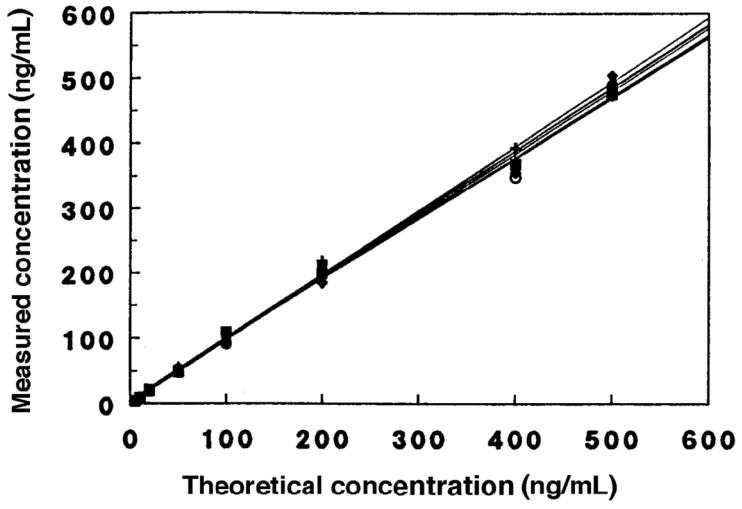

Figure 6. Calibration curves for HC and the IS.

Precision

Method precision was assessed using data from four analysis replications of three reference standards on each of three days. The three levels of these standards were the low, middle, and upper values of the calibration range. The concentrations were 15, 75, and 300 µg/L for the analyte. The intra-assay (intraday) precision was determined at each level within each day by calculating the %RSD of the found concentrations. The interassay (interday) precision was determined at each level by calculating the %RSD of the found concentrations from all three days combined (%RSD from all runs). As Table I indicates, the intra-assay precision ranged from 2.2 to 8.4 over the three levels. The interassay precision results were 7.2, 5.0, and 5.2 for 15, 75, and 300 ng/mL, respectively. Clearly, there was no significant difference between the intra- and interassay precision, at least over the limited 3-day period investigated here.

Accuracy

Method accuracy was assessed by spiking synthetic urine with known amounts of the analyte and IS. The analyte was spiked into urine at the levels of 15 and 300 ng/mL. The data from these fortified samples were compared with neat (standard) nonextracted samples. The accuracy of these found values were 97% and 96% recoveries for 15 and 300 ng/mL, respectively.

Table I. Within- and Between-Day Precision for HC at Three Different Levels

	%RSD* (n = 6) for the given level (ng/mL)		
Day	15	75	300
1	4.9	2.8	4.6
2	8.4†	4.7	6.4
3	6.1	5.1	2.2
Interday‡	7.2	5.0	5.2

* Based on the peak-height ratio.
† n = 5.
‡ All runs for each level.

Table II. Within- and Between-Freeze–Thaw Cycle Precision for HC at Three Different Levels of Urine QCs

	%RSD* (n = 3) for the given level (ng/mL)		
Cycle	15	75	300
1	6.4	1.8	2.6
2	3.3	4.5	3.2
3	4.8	2.5	4.2
Interday†	5.0	3.1	3.7

* Based on the peak-height ratio.
† All nine runs for each level.

Table III. Comparison of Results from the Dilute-and-Shoot and LLE Techniques for Three Unknown Samples

	Ratio of HC and the IS	
Sample	Dilute-and-shoot	LLE
S1	0.118	0.118
S2	0.117	0.127
S3	0.0646	0.0681

Ruggedness

Ruggedness is defined as a measure of reproducibility of the test results from analyst to analyst. In this study, both the sample preparation and LC–MS–MS analysis were done by different analysts. Our finding that analysis results were highly reproducible implies that both the sample preparation and LC–MS–MS method were rugged. We also checked the possibility of sample carryover from a previous injection using the following analysis sequence: a synthetic urine blank, a blank spiked at 100 ng/mL of the analyte and IS, a blank spiked with only the IS, a blank spiked with only the analyte, and a nonspiked blank. No peak was observed in the final nonspiked blank reaction mass, indicating that there was no carryover from the previous run.

System repeatability

In this study, the system repeatability of the LC–MS–MS method was determined by running over 50 samples at 200 ng/mL. The average peak-height ratios of the analyte relative to the IS, standard deviation, and %RSD were found to be 201.3, 6.9, and 3.4, respectively, and the retention time %RSD was 1.7.

Stability of solutions

The effect of freeze–thaw cycles and benchtop stability of the analyte in solution were investigated by analyzing a spiked sample immediately upon preparation and on subsequent days of the anticipated storage period. Table II shows the within- and between-freeze–thaw cycle precision for our analyte at the three levels of plasma quality previously described. The %RSD was calculated on the basis of peak-height ratio and was 3.3–6.4, 1.8–4.5, and 2.6–4.2 for 15, 75, and 300 ng/mL, respectively. These results show that the solutions used here were fully stable through the freeze–thaw cycles.

System suitability

System suitability tests were based on the concept that the equipment, electronics, analytical operations, and samples constituted an integral system that could be evaluated as a whole. System suitability was used to ensure system performance before or during the analysis of unknowns using the reproducibility of replicate injections, peak tailing factor, resolution, and peak retention time as the criteria. These parameters were evaluated throughout the analysis. Prior to analysis, we ran a calibration mix in order to check system performance. Our control samples consisted of QC and a mixture of main components. The results of our study indicated that this system is highly suitable.

Considerations for the dilute-and-shoot technique

Table III shows a comparison of results from the dilute-and-shoot and liquid–liquid extraction (LLE) techniques for three unknown samples. This experiment was done to reveal whether there was any depression of the signals because of salt in the urine samples and check for any interference from other analytes when the dilute-and-shoot technique was used. Our results showed that both techniques gave an identical response ratio for HC and the IS.

Conclusion

This study shows that LC–MS–MS can provide a rapid, relatively easy-to-use approach for the bioassay of HC in human urine without compromising assay sensitivity. This method overcame major disadvantages of previous methods involving stripped biomatrices; it is easier and cheaper to perform and the quantitative results are more reliable. The method requires little sample preparation other than dilution with water. The analysis time is short (the analytes of interest were obtained within 2.3 min). For the analysis of both the analyte and IS, the method has been thoroughly validated. The LOQ of the analyte was 5 ng/mL using APCI, and separations were robust. The selectivity of this method has shown to be excellent, with no interference from other analytes. The results obtained using this method showed that the interday precision for the analyte was less than 7.5% RSD. Furthermore, throughout our study, the HPLC column used for this work remained stable.

Acknowledgments

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