Spectrophotometric Determination of Paracetamol in Urine with Tetrahydroxycalix[4]arene as a Coupling Reagent and Preconcentration with Triton X-114 Using Cloud Point Extraction

Hayati FILIK,*,[a] İzzet ŞENER,[b] Sema Demirci CEKIÇ,[a] Emine KILIÇ ,[c] and Reşat APAK[a]

[a] *Department of Chemistry, Faculty of Engineering, Istanbul University; Avcilar, 34320, Istanbul, Turkey:* [b] *Department of Chemistry, Faculty of Science and Arts, Pamukkale University; 20017, Denizli, Turkey: and* [c] *Department of Chemistry, Faculty of Science, Ankara University; 06100, Ankara, Turkey.* Received December 5, 2005; accepted February 23, 2006

In the present paper, conventional spectrophotometry in conjunction with cloud point extraction-preconcentration were investigated as alternative methods for paracetamol (PCT) assay in urine samples. Cloud point extraction (CPE) was employed for the preconcentration of *p*-aminophenol (PAP) prior to spectrophotometric determination using the non-ionic surfactant Triton X-114 (TX-114) as an extractant. The developed methods were based on acidic hydrolysis of PCT to PAP, which reacted at room temperature with 25,26,27,28-tetrahydroxycalix[4]arene (CAL4) in the presence of an oxidant (KIO_4) to form an blue colored product. The PAP-CAL4 blue dye formed was subsequently entrapped in the surfactant micelles of Triton X-114. Cloud point phase separation with the aid of Triton X-114 induced by addition of Na_2SO_4 solution was performed at room temperature as an advantage over other CPE assays requiring elevated temperatures. The 580 nm-absorbance maximum of the formed product was shifted bathochromically to 590 nm with CPE. The working range of 1.5—12 μg ml^{-1} achieved by conventional spectrophotometry was reduced down to 0.14—1.5 μg ml^{-1} with cloud point extraction, which was lower than those of most literature flow-through assays that also suffer from nonspecific absorption in the UV region. By preconcentrating 10 ml sample solution, a detection limit as low as 40.0 ng ml^{-1} was obtained after a single-step extraction, achieving a preconcentration factor of 10. The stoichiometric composition of the dye was found to be 1 : 4 (PAP : CAL4). The impact of a number of parameters such as concentrations of CAL4, KIO_4, Triton X-100 (TX-100), and TX-114, extraction temperature, time periods for incubation and centrifugation, and sample volume were investigated in detail. The determination of PAP in the presence of paracetamol in micellar systems under these conditions is limited. The established procedures were successfully adopted for the determination of PCT in urine samples. Since the drug is rapidly absorbed and excreted largely in urine and its high doses have been associated with lethal hepatic necrosis and renal failure, development of a rapid, sensitive and selective assay of PCT is of vital importance for fast urinary screening and antidote administration before applying more sophisticated, but costly and laborious hyphenated instrumental techniques of HPLC-SPE-NMR-MS.

Key words paracetamol determination; spectrophotometry; cloud point extraction; *p*-aminophenol; calix[4]arene; urine

Paracetamol (*N*-acetyl-*p*-aminophenol; abbreviated as PCT) has been widely used as analgesic and antipyretic drug. Paracetamol is a weak acid (pK_a=9.5) which is rapidly absorbed and distributed after oral administration and excreted largely in urine: 45—55% as glucuronide conjugates, 20—30% as sulphate, 15—55% as cysteine and mercapturic acid conjugates and only 1—5% unchanged.[1] Although PCT is usually well tolerated when used at the recommended dose, large doses have been associated with lethal hepatic necrosis and renal failure.[2] Various methods have been proposed for the determination of PCT in biological fluids (urine, plasma and serum). Relatively few reports describe high-performance liquid chromatographic (HPLC) methods for the estimation of urine and serum concentrations of PCT using spectrophotometric,[2—10] fluorescence[11] and electrochemical detection.[12] Other methods such as nuclear magnetic resonance (NMR),[13] capillary electrophoresis (CE),[14] and gas chromatography (GC)[15] have also been reported. There are many reliable methods for assaying urinary PCT levels, but they are often time-consuming, technically demanding, and requires the use of costly, highly specialized instruments.

In the first practical spectrophotometric method developed,[16] PCT was extracted with ether and hydrolyzed with acid to *p*-aminophenol, which was then coupled with phenol in the presence of sodium hypobromite to form an indophenol dye. Since then, other spectrophotometric methods for PCT assay in biological fluids have been described.[17—25] The majority of published spectrophotometric methods are based on ring-nitration,[23] diazotization,[17—19] differential absorbance measurement,[18] direct acid reduction,[21] and oxidative coupling with some phenolic reagent to form an indophenol dye.[24,25] Generally, urinary PCT is not directly assayed, and requires preliminary hydrolysis of PCT to *p*-aminophenol (abbreviated as PAP) for final determination. Urinary screening of PCT is mostly based on acidic[25—30] or enzymatic[31—33] hydrolysis of PCT to PAP, the latter requiring 17—20 h. In most of the conventional spectrophotometric procedures, the hydrolysis product (PAP) reacts with a special chromogenic reagent in basic medium to form an indophenol blue dye. A number of chromogenic reagents including phenol,[30] *o*-cresol,[25,26,28] *o*-xylenol,[34] resorcinol,[31] and 8-hydroxyquinoline[35] have been utilized. Welch and Conney[30] have used the method of Brodie and Axelrod[16] for the determination of PCT in urine samples by eliminating the extraction of PCT or PAP into an organic solvent. According to Heirwegh and Fevery,[29] in strongly acidic medium, PAP resulting from differential extraction and acid hydrolysis of total PCT in urine is diazotized and the diazonium salt cou-

* To whom correspondence should be addressed. e-mail: filik@istanbul.edu.tr © 2006 Pharmaceutical Society of Japan

pled with *N*-(1-naphthyl)ethylenediamine in the presence of ethanol to a colored product useful for colorimetry. Recently, automated methods were used for such reagent based colorimetric detections. Criado *et al.*,[28] Chen *et al.*,[34] Bocxlaer *et al.*[31] and Morris *et al.*[35] developed a fully automated urinary screening system for the quantitative determination of paracetamol. Although it was reported in Bocxlaer's paper[31] that *p*-aminophenol is easily oxidized to *p*-iminoquinone forming the basis of such assays, coupling with resorcinol in alkaline medium to convert into an indophenol dye would naturally yield higher absorptivity and lower detection limits. Parojčić *et al.* described the use of second-order derivative UV spectrophotometry for direct determination of PCT in urine without preliminary extraction or prior derivatization.[36] Criado *et al.* have investigated the use of an evaporative light scattering detector (ELSD) for the determination of PCT in urine samples.[37] However, most of these methods have detection limits close to the urinary levels of PCT while urinary clearance of the drug after a certain time may reduce the concentrations to such low levels that preconcentration/detection of the toxic agent may be necessary. Up to now, no cloud point extraction-preconcentration technique with spectrophotometric detection of PAP or PCT in urine samples have been reported in the literature.

In our previous study,[38] we developed a method for the sequential spectrophotometric determination of PCT and PAP in pharmaceutical products, which had the advantage of a lower detection limit than those of other similar methods. The aim of this work is to develop a new spectrophotometric method with CPE preconcentration for PAP by the use of 25,26,27,28-tetrahydroxycalix[4]arene (CAL4) as a coupling agent. Visible spectrophotometry was preferred because of its simplicity, low cost and rapid analysis, wheras cloud point extraction (CPE) was adopted for obtaining higher sensitivitiy and lower detection limits. Calix[4]arenes, the well-known family of macrocyclic ligands, frequently serve as molecular scaffold for the construction of more elaborate supramolecular systems, such as various receptors possessing interesting complexation abilities towards target molecules or ions.[39,40] The developed spectrophotometric method involves oxidative coupling of PAP with CAL4 containing an electron-donating group, based on an electrophilic aromatic substitution reaction catalyzed by potassium periodate. Such aromatic substitution reactions generally require phenolic compounds (exemplified with *o*-cresol, *o*-xylenol, resorcinol, and phenol, from the literature). On the other hand, preconcentration efforts using surfactants with high cloud point temperatures may lead to the decomposition of the analyte. Hence, these phenolic coupling agents may be unsatisfactory in the course of CPE-preconcentration of PAP or PCT. In the CPE method, surfactant TX-114 was used as the extractant and a Na_2SO_4 solution was used for promoting phase separation. The indophenol blue dye formed was subsequently entrapped in the micelles of the surfactant. The condensed surfactant phase containing the PAP-CAL4 dye enabled the quantitation of PAP by direct spectrophotometry after CPE, and the developed method was applied to PCT assay in urine.

Experimental

Apparatus Spectrophotometric studies were performed on a Cary-1E UV–visible spectrophotometer. Elemental analysis results were obtained from a Thermo Finnigan Flash EA 1112 model analyser.

Reagents All chemicals used were of analytical reagent grade. PAP was obtained from E. Merck. PAP stock solution was prepared by dissolving 109 mg of PAP in 100 ml of ethanol. PCT stock standard solution (1×10^{-3} mol l^{-1}) was prepared by dissolving pure PCT in 100 ml of distilled water using an ultrasonic water bath to ensure complete dissolution. Working solutions at lower concentrations were freshly prepared by serial dilution with H_2O. 25,26,27,28-Tetrahydroxycalix[4]arene (CAL4) was synthesized according to the method described in literature.[41,42] The structural formula of CAL4 coupling ligand is depicted in Chart 1. A CAL4 solution (1×10^{-3} mol l^{-1}) was prepared by dissolving the required amount of CAL4 in 0.01 M NaOH. The solution is stable in the refrigerator for several weeks. The ligand solution is colorless in alkaline solution.

Sample Preparation Analysis of Urine Samples: Urine was collected at timed intervals from one healthy volunteer following oral PCT administration. The conventional batch hydrolysis of urine samples containing PCT was carried out as follows: A 1 ml sample of urine was treated with 2 ml of 4 M HCl in a beaker, mixed well, and then placed in a boiling water-bath for 30 min. After cooling to room temperature, the hydrolyzed sample was neutralized and diluted (100-fold) to volume with distilled water. An aliquot of the analyte was taken and assayed according to the general procedures.

Procedures for Calibration Curves. Procedure A (Direct Spectrophotometric Determination) Appropriate volumes of standard and/or unknown PAP or hydrolyzed PCT sample solutions (1×10^{-5}—8×10^{-5} M) were transferred to a 5 ml volumetric flask, and 1 ml of 1×10^{-3} M CAL4, 1 ml of 1×10^{-3} M KIO_4 solution, 1 ml of 1% Na_2CO_3 solution were added. The order of addition of reagents was not important. The color was developed in alkaline solution. The mixture was shaken thoroughly for 30 s, and the volume was completed to the mark with water. The mixture solution was allowed to stand for a further 10 min at room temperature. Finally, the absorbance of the resulting solution was measured at $\lambda=580$ nm against the reagent blank. This developed method is recommended for the determination of 1.5—12 μg ml^{-1} paracetamol. The blue dye utilized in colorimetry was stable for more than 2 d, and the results were reproducible. With *o*-aminophenol, however, a yellow color was obtained; *m*-aminophenol gave only a very slight pink color.

Procedure B (Cloud-Point Extraction Preconcentration Procedure) A CPE-preconcentration technique with spectrophotometric detection was developed. Appropriate volumes of standard or unknown PAP sample solutions (1×10^{-6}—1.0×10^{-5} M) were transferred to a *ca.* 10—15 ml centrifuge tube, and 1 ml of 1×10^{-3} M CAL4, 1 ml of 1×10^{-3} M KIO_4 and 1 ml of 1% Na_2CO_3 solution were added. The color did not develop instantaneously at room temperature, and after staying for 10 min, an almost constant absorbance was obtained. To this mixture, 1 ml of 2% TX-114 solution as the surfactant and 2 ml of 5% (w/v) Na_2SO_4 were added, and the final volume was completed to the mark with distilled water. The mixture was clouded with the addition of Na_2SO_4 and the assay was performed at 25 °C. Then the resulting turbid solution was centrifuged without a need for incubation. Separation of the phases was achieved by centrifugation for 5 min at 5000 rpm. The mixture was cooled down in an ice-water bath in order to increase the viscosity of the surfactant-rich phase. The bulk aqueous phase was easily decanted. For absorbance measurements, the resulting blue colored micellar phase was dissolved with ethanol and diluted to required volume before determination (preferably 1 ml). Finally, the absorbance of the resulting solution was directly measured at $\lambda=590$ nm against a reagent blank containing all chemicals (used in the method) but PAP. This CPE method allows the determination of low concentrations of PCT in the range 0.14—1.5 μg ml^{-1}.

Results and Discussion

Characteristics of the Reaction The proposed reaction of PAP with CAL4 as the basis of spectrophotometric PCT assay is depicted in Chart 1. According to this reaction scheme, PAP produces a reactive benzoquinoneimine in the presence of KIO_4 in alkaline medium, which is then coupled to CAL4 to produce a blue derivative exhibiting maximum absorbance at 580 nm (Fig. 1). In the presence of surfactant, the PAP-CAL4 dye shows a bathochromic shift, and measurements are carried out at 590 nm. The stoichiometric composition of indophenol dye was studied under the established conditions by the molar ratio method, and was found to be PAP : CAL4 = 1 : 4. To investigate the stability of PAP-CAL4 dye in TX-114 medium, the absorbance of the dye was meas-

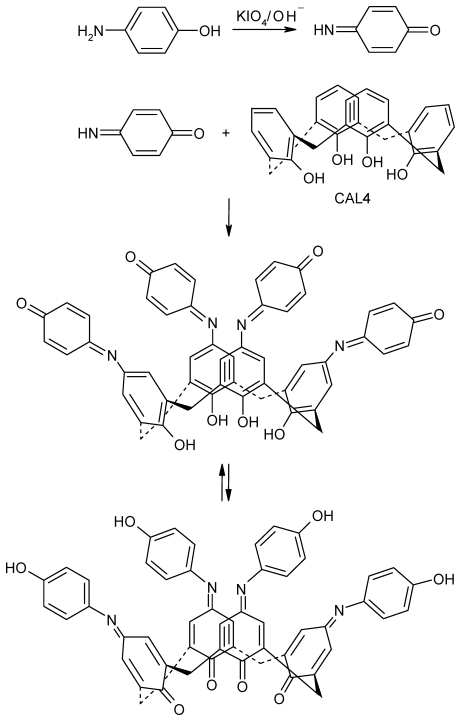

Chart 1. Reactions Involved in the Proposed Method for PAP Determination

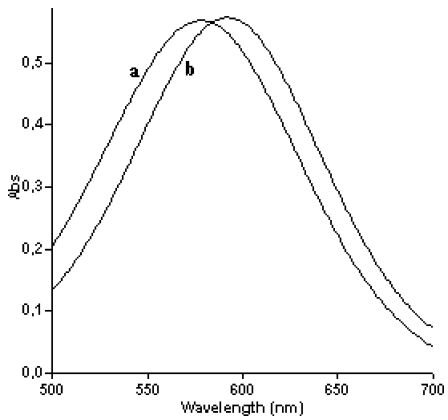

Fig. 1. Absorption Spectrum of the Reaction Product with and without TX-114

$C_a=4\cdot10^{-4}$ M ($\lambda_{max}=580$ nm; without extraction), $C_b=5\cdot10^{-6}$ M ($\lambda_{max}=590$ nm; with extraction).

ured as a function of time. The blue derivative with and without extraction was stable for more than 2 d and the results were reproducible. Most colored products reported to form in other methods were stable for 1—3 h. The combined CPE-preconcentration and spectrophotometric PCT assay is a useful alternative that can compete with most of the spectrophotometric methods available in literature. In comparison with the performance of other methods, approximately 2.5—50 times lower detection limits were achieved, and the analytical figures of merit of the proposed and literature methods are shown in Table 1.

Effect of Experimental Conditions The effect of concentration of CAL4 ligand on the extraction was studied. Different molar excess ratios of CAL4 were added to the solution which has a constant PAP concentration, and absorbance was measured with the aid of the general procedure. The results obtained show that the absorbance increases with increasing CAL4 ligand concentration from 1×10^{-3} to 2×10^{-3} mol l^{-1}. For CAL4 concentrations higher than 2×10^{-3} mol l^{-1}, the absorbance remains almost constant. According to these results, a CAL4 amount of 2 ml of 1×10^{-3} mol l^{-1} was used in all subsequent work. The effect of KIO_4 concentration was examined in the range 1×10^{-3}—1×10^{-2} mol l^{-1}. The absorbance decreases by increasing KIO_4 concentration. However a slight excess of the oxidant is required to cope with possible interferences of reducing agents such as *N*-acetylcysteine and ascorbic acid.

On the other hand, under the proposed experimental conditions, optimal CPE temperature and time are necessary to complete the reactions. Several concentrations of the two surfactants used in this study, namely TX-100 and TX-114, were tested in order to ensure maximum extraction efficiency. TX-114 was selected for the formation of the surfactant rich phase because of its very low cloud point temperature, which facilitates phase separation without incubation at room temperature. The surfactant TX-100 has a relatively high cloud point, around 70 °C, and requires 10 min incubation time at elevated temperature (see Table 2). The effect of the equilibration temperature was examined over the range 25 to 50 °C. The extraction efficiency remains constant in the temperature range from 25 to 50 °C. So, the equilibration temperature was selected as 25 °C, which is a very important advantage of the proposed method. In addition, the effect of centrifugation time upon extraction efficiency was examined for the range 1—10 min. Complete phase separation was achieved for time periods ≥3 min. A centrifugation time of

Table 1. Comparison of the Figures of Merit of Ten Spectrophotometric Methods for the Determination of PAP in Urine Samples

Method	Sample	Detection limit (ng ml^{-1})	Linear range (μg ml^{-1})	Reference
Phenol	Urine	NR	100—800	Welch *et al.*[30]
o-Cresol	Urine	100	0.3—12	Criado *et al.*[28]
Xylenol	Urine	NR	20—400	Chen *et al.*[34]
Resorcinol	Urine	900	2—100	Bocxlaer *et al.*[31]
8-Hydroxyquinoline	Urine	NR	0—2.5	Morris *et al.*[35]
Derivative UV	Urine	2000	5—30	Parojčić *et al.*[36]
Diazotisation	Urine	NR	2—10	Heirwegh *et al.*[29]
ELSD	Urine	300	1—100	Criado *et al.*[37]
UV–visible	Urine	500	1.5—12	This work
CPE-UV–visible	Urine	40	0.14—1.5	This work

NR: Not reported.

Table 2. Optimization of Cloud Point Extraction Variables in CPE-Spectrophotometry

Variable	Range tested	Selected value
CAL4 (mol l^{-1})	1×10^{-3}—2×10^{-3}	2 ml of 1×10^{-3}
KIO_4 (mol l^{-1})	1×10^{-3}—1×10^{-2}	1 ml of 1×10^{-3}
Na_2CO_3 (% w/v)	1.0—2.0	1 ml, 1% soln.
Triton X-100 (% w/v)	1.0—5.0	2 ml, 4% soln.
Incubation temperature (°C)	60—100	≥80 °C
Incubation time (min)	5—20	10 min[a)]
Centrifugation time (min)	1—5	5 min
Triton X-114 (% w/v)	1.0—5.0	1 ml, 2% soln.
Incubation temperature (°C)	25—50	25 °C
Incubation time (min)	—	None[b)]
Centrifugation time (min)	1—5	5 min

a) Triton X-100 required 10 min incubation at elevated temperature. *b*) Triton X-114 did not require incubation.

5 min was selected as suitable.

The cloud point temperature depends on the structure of the surfactant and its concentration. For that reason, the effect of the concentration of TX-114 on the absorbancy of the system was examined. Quantitative CPE was observed for a surfactant solution volume ≥1.0 ml. In subsequent experiments, the volume of TX-114 solution was fixed at 1 ml. In the case of most non-ionic surfactants, the presence of inert salts may facilitate phase separation by increasing the density of the aqueous phase and by possible 'salting-out effects' familiar to the theory of solvent extraction. When the salt concentration is increased, the aggregation number increases and the critical micelle concentration remains constant. Consequently in this work, Na_2SO_4 solution was used for promoting phase separation. A 2 ml of 5% (w/v) Na_2SO_4 solution is recommended to induce cloud-point phase separation in all analyses. The selected chemical parameters are listed in Table 2.

Effect of Sample Volume and Concentration Factor The influences of the sample volume on the CPE of PAP were also examined. A 5.5 μg amount of PAP from different volumes of aqueous phase was extracted with CAL4 into 1 ml of 2% TX-114 by the recommended procedure. The absorbance of the surfactant rich phase was found to remain constant when the volume of aqueous phase was increased up to 15 ml. Quantitative recovery was made (>95%) up to 15 ml of sample volume, beyond which the recovery decreased gradually. For that reason, 10 ml of sample solution was used in further work. The concentration factor (CF), defined as the ratio of the volume of solution used to that of the surfactant rich phase assuming quantitative recovery of the analyte into micellar phase,[43,44)] was found as 250. Because of dilution of surfactant-rich phase, the experimental concentration factor was found to be 10 fold.

Calibration Graph Procedure A: For direct spectrophotometry, Beer's law was obeyed over a PAP concentration range 1×10^{-5}—8×10^{-5} M. The linear regression equation was : $A_{580\,\mathrm{nm}}=1.4\times10^4C-0.01$. Molar absorptivity at 580 nm was 1.4×10^4 l mol^{-1} cm^{-1} and the correlation coefficient $r=0.9998$.

Procedure B: For CPE-preconcentration and spectrophotometry, a calibration graph was constructed by preconcentration of 10 ml of sample standard solutions with TX-114. The regression curve obtained was reasonably linear and can be expressed by the following equation: $A_{590\,\mathrm{nm}}=8.2\times10^4C+0.01$ ($r=0.9989$) where A is the absorbance at 590 nm and C is the molar concentration of PAP in the final solution. The absorbance was linear for 1×10^{-6}—1.0×10^{-5} mol l^{-1} of PAP. The limit of detection (LOD) achieved for PAP, estimated by $3s_B/m$ (where s_B is the standard deviation of 10 measurements of the blank and m is the slope of the calibration line), was 0.5 μg ml^{-1} for the case without extraction and 40 ng ml^{-1} for with extraction. LOQ was 0.13 μg ml^{-1} using CPE-spectrophotometry. The reproducibility of the method expressed as RSD was checked on five individual samples containing 0.55 μg ml^{-1} of PAP and a corresponding concentration of PCT. The relative standard deviation for synthetic solutions was found to vary between 0.5 and 1.0% for PAP. The intra- and inter-assay CVs of urine samples collected from a PCT-administered patient (up to 15 h of sampling time) were 1.5 and 2.7%, respectively.

Interference Studies The influence of foreing compounds and excipients that can be found in pharmaceutical formulations containing paracetamol was also studied for both methods. Acetylsalicylic acid, caffeine, codeine, saccharin sodium salt, aspartame, bicarbonate sodium salt, benzoate sodium salt, maleate, sorbitol, procaine, *p*-aminobenzoic acid, sulfacetamide, and atropine did not interfere. All the compounds studied at concentrations up to 100 μg ml^{-1} caused no interference in the determination of 1.0 μg ml^{-1} of paracetamol. The main interferent compounds were only ascorbic acid and citric acid. These compounds decreased the absorbance sharply (at an interferent ratio of 10 : 1). However, this is not a serious inconvenience of the method, as the concentration of these compounds in urine is lower than the tolerated ratio.[28)]

Application of the Assay Under optimal experimental conditions, no color was observed with the intact PCT solution. On the other hand, the assay was utilized for the analysis of urine samples from a healthy volunteer administered a single 500 mg dose of PCT. Samples of urine from volunteer was collected at approximately 5 h intervals for 20 h. One milliliter urine samples were diluted 100-fold, hydrolyzed, and analyzed using the proposed direct and extraction methods. The results obtained by both methods were at the same level as earlier findings of Criado *et al.*[28)] The performance of the proposed methods was compared statistically to that of the literature method[38)] in terms of Student's *t*-test and the variance ratio *F*-test. At 95% confidence level, the calculated *t*- and *F*-values did not exceed the theoretical *t*-value was 2.776 (for $N=5$) and *F*-value was 6.39 (for $N=5$) for all sampling times tested, confirming validation of both methods (see Table 3). For spiked (with 25 μg PCT) and rediluted urine samples up to a sampling time of 15 h, the recoveries were 100±3%. After 20 h of urinary clearance, the recoveries were between 100 and 110%.

Requirement for a Novel Spectrophotometric Method and Advantages over Other Procedures Naturally, hyphenated techniques such as LC-SPE-NMR-MS can selectively and sensitively detect PCT and its metabolites in physiological fluids.[45)] However, LC-MS may only become a gold standard in clinical and forensic screening of drug residues if, at a later date, the costs of the apparatus is markedly reduced and the current disadvantages like irreproducibility of fragmentation, reduction of ionization by matrix, *etc.* are

Table 3. Determination of PCT with Developed and Literature[38] Methods in Human Urine Collected at Approximately 5 h Intervals for 20 h

Sampled time (h)	Proposed Method A	Proposed Method B	Comparative method	Added (μg ml^{-1})	Total found (μg ml^{-1}) A	B
5	180.5±1.5 F=1.96 t=1.52	181.0±2.2 F=1.10 t=1.29	181.6±2.1	20	201±2	199±3
10	70.1±2.1 F=1.19 t=2.22	69.5±1.5 F=2.35 t=1.84	70.5±2.3	20	91±2	92±3
15	27.2±2.8 F=1.48 t=1.84	27.8±1.7 F=2.04 t=2.28	27.3±2.3	20	47±1	46±2
20	8.3±1.9 F=1.73 t=2.42	8.6±1.6 F=2.44 t=1.45	8.5±2.5	20	29±2	32±2

N=5 for each determination, the critical values of t and F being 2.776 and 6.39, respectively; samples collected from patient administered a single dose of 500 mg PCT. The urine samples (1 ml) were diluted 100-fold; normal and spiked samples were simultaneously analyzed.

overcome.[46] Aside from the major barriers of initial capital outlay for instrumentation and lack of suitably trained scientists to fast screening of biological fluids for drug residues, the major challenges of HPLC-MS may be summarized as ease of use and automation, interpatient variability in relation to matrix effects, availability of suitable internal standards, and harmonization of methods to meet regulatory requirements.[47]

The developed method is a simple, rapid, and reasonably specific procedure for the parent drug applicable to emergency cases of toxicity where chromatographic procedures would not be practicable, particularly at night.[48] Specificity and rapidity are required to establish the toxic agent and immediately guide the clinician in the administration of sulfhydryl compounds.[45] The preliminary hydrolysis of PCT to PAP may be accelerated with the aid of a microwave oven.[27,28] Considering the lower hydrolysis efficiency of PCT under alkaline conditions, acidic conditions were selected considering that a higher hydrolysis efficiency would allow a smaller sample volume.[28] Prior conversion of PCT to PAP that ends up with a colored species is useful because most direct flow-through UV methods suffer from non-specific absorption and therefore cannot be applied to physiological fluids.[49] Only simpler matrices such as pharmaceutical preparations could be assayed for PCT with such flow-through sensors.[50—52] The current method is advantageous over the diphenylpicrylhydrazyl radical method where the latter reagent has to be prepared for each run[18] and reacts with other plasma antioxidants. With a similar reasoning, the Fe(III)-tripyridyltriazine, also known as the FRAP reagent in total antioxidant assays of foodstuffs and body fluids, has been used for PCT assay in biological fluids[21] such as serum and plasma, but this reagent would also be affected from other serum antioxidants,[53] and therefore would not be specific for paracetamol. The proposed PCT assay is not interfered by a number of common drug ingredients (with the exception of PAP) such as acetyl salicylic acid, procaine, *p*-aminobenzoic acid, sulfacetamide, and atropine, as reported in other coupling assays with phenolic agents.[28] Here it should be noted that most literature methods report false negatives in the presence of *N*-acetylcysteine, *i.e.*, the drug choice for treating PCT overdose in order to combat nephrotoxic and hepatotoxic side effects[23]; the developed method effectively reports the correct value for PCT (in the presence of 40-fold *N*-acetylcysteine) in a slightly extended time period (compared to that of the recommended method), because KIO_4 is used as an auxiliary oxidant in coupling rather than molecular oxygen alone. Otherwise, the false-negative results observed in the urinary application of the method of Simpson and Stewart[24] probably caused by *N*-acetylcysteine or ascorbic acid should be overcome by the addition of other chemicals such as $CuSO_4$[25] most likely acting as an oxidant. Nevertheless the concentration of *N*-acetylcysteine in urine, found after administration of PCT overdose treatment, is usually lower than the tolerated ratio of the resorcinol-coupling method,[28] but tolerance of the proposed method to such sulfhydryl agents should still be considered as an advantage. Another superiority to certain phenolic coupling methods requiring catalysis is that the reagent blank is not colored as that of the *o*-cresol method using the $Cu(NH_3)_4^{2+}$ as catalyst,[54] and that there is no manganese oxyhydrate precipitate formation in a homogeneous solution.[31] Although the proposed method seems similar to other PAP assays reported in literature based on the formation of an indophenol dye in alkaline medium with a phenolic reagent, the extent of conjugation (rearrangement of alternating double bonds) in the final colored product (see Chart 1) is much deeper, thereby enhancing the absorptivity and sensitivity. Calixarenes are a class of phenolic metacyclophanes possessing a versatile p-electron rich cavity,[55] thereby serving the purpose of the developed method. As a result, only PAP (but not *o*- or *m*-aminophenol) reacts with CAL4. An increase in sensitivity of conventional methods is desirable, because approximately 89% of PAP is eliminated from urine within 24 h postexposure,[31] and only a small fraction of the drug may remain in urine after a certain time for toxicity evaluation. The fact that color development is relatively fast in alkaline solution is important to hinder autoxidation of tetrahydroxycalix[4]arene reagent—as reported for a number of other phenolic coupling agents[31]—and to ensure colored product formation. The working range of 1.5—12 μg ml^{-1} achieved by conventional spectrophotometry developed in this work was reduced down to 0.14—1.5 μg ml^{-1} with cloud point extraction. Carry out of CPE preconcentration at room temperature is another advantage of the developed assay. A low detection limit allows a high dilution of the samples thus reducing po-

tential interferences from the sample matrix (mainly from protein degradation through urine hydrolysis[28]). The current method neither suffers from the false-positive results encountered in ring-nitration spectrophotometric methods nor requires solvent extraction-separation with the highly flammable diethyl ether used to overcome such a difficulty.[23] Prior separation of salicylic acid or acetylsalicylic acid with diethyl ether extraction is also necessary for spectroflurimetric assays of PCT.[56] The long-wavelength absorption maxima of the original and CPE-preconcentration spectrophotometric methods at 580 and 590 nm, respectively, hinder possible interferences of other biological materials which may absorb at shorter wavelengths. The method may also be used for biological monitoring of human exposure to the carcinogenic aniline, because absorbed aniline is extensively oxidized to PAP which is excreted in urine.[31]

Conclusions

The proposed CPE-spectrophotometric method has proved to be specific for PAP and can be applied to the determination of PAP or PCT in various pharmaceutical, environmental and biological fluids such as urine. Use of calix[4]arene as an auxiliary ligand for indophenol dye formation from PAP, achievement of a higher molar absorptivity—and thus sensitivity—than those of most spectrophotometric methods, direct applicability to urinary PCT assay unlike most UV flow-through methods interfered by urinary constituents, and use of the developed photometric method with CPE-preconcentration using the nonionic surfactant triton X-114 at room temperature constitute the original contributions of the proposed method.

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