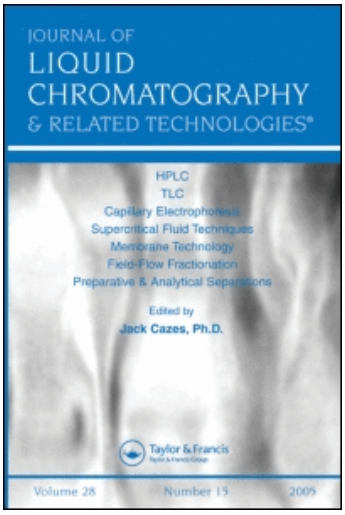

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EVALUATION OF SOLID PHASE EXTRACTION PROTOCOLS FOR ISOLATION OF ANALGESIC COMPOUNDS FROM BIOLOGICAL FLUIDS PRIOR TO HPLC DETERMINATION

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ABSTRACT

A comparative study of various sorbents, reversed phase silica based C_{18}, C_8, and copolymeric hydrophilic-lipophilic balanced, from different manufacturers and various eluting solvents (methanol, acetonitrile, and isopropanol) was conducted for optimization of isolating the constituents of multi-component analgesic mixtures by means of Solid Phase Extraction (SPE). Optimized SPE protocols were subsequently applied to human serum and urine samples. Traditional minicolumns and disc formats of C_{18} sorbent were studied as well. The effect of sorbent bed conditioning was also investigated when using novel copolymeric sorbent materials, such as OASIS and NEXUS, as

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both claim to function under no conditioned sorbent bed as being water wettable.

An analgesic mixture containing paracetamol, caffeine, and codeine was selected as a model for this survey, since they very often co-exist in pharmaceutical formulations. Analytes were monitored at 240 nm, after isocratic elution from a C_8 Inertsil analytical column. The mobile phase was a mixture of methanol and ammonium acetate (0.05 M) at a volume ratio of 40:60. Statistical evaluation revealed satisfactory accuracy, repeatability, and intermediate precision. Pharmaceutical formulation analysis yielded high recoveries ranging from 95.4 to 107.5%. Various recovery rates were obtained when the different protocols were applied. Reversed phase C_{18} sorbent yielded a 80–90% recovery, while copolymeric sorbents reached the 100% of analyte concentration in optimizing extraction conditions concerning the activation step and the eluting solvent.

INTRODUCTION

The need for fast and efficient analytical procedures within the pharmaceutical sector is rapidly growing. The pre-analytical phase or sample processing step is often the bottleneck step in any analytical method, as it is the most error-prone part. Sample preparation in bioanalysis is aimed at an adequate reduction in matrix interference. Typical biological samples include several matrices: blood plasma, serum, whole blood, urine, saliva, hair etc. Among them, blood serum and urine are the most popular in clinical analysis. Urine is one of the most commonly studied, particularly because of its relative ease of collection, and because it is nearly universal as a means of excretion of parent drug compounds, metabolites, or both. As a matrix, it has moderate complexity, and typically contains both organic and inorganic constituents, as well as a relatively high variability. Plasma samples contain a significant amount of salt and proteins that can be precipitated or adsorbed on reversed phase packings. The adsorbed protein can easily foul the column, resulting in changes in the separation and ultimately clogging the column.

Although packings have been designed for the direct injection of plasma samples, most analyses are performed using classical reverse phase packings; therefore, if one desires a reasonable column life, sample preparation is unavoidable. Plasma is prepared from whole blood that has been treated with an anti-clotting substance such as heparin. It is the supernatant that results when the cellular components of blood are removed by centrifugation. Serum is the straw-colored liquid that separates from the clot that forms in whole blood. A method

developed for plasma can normally be applied without modification to serum. Much of the discussion for analysis of drugs from plasma also applies to analysis from whole blood (1).

The techniques used in bioanalysis for sample preparation are sample dilution, protein precipitation, or extraction of the analyte from the matrix. Sample dilution can occasionally be applied if the analyte is present in such a high concentration that the matrix interferences can adequately be diluted, with the analyte still being detectable. The direct injection method is the simplest and most rapid technique using deproteinization of plasma with an organic modifier such as acetonitrile, or an acidic solution such as perchloric acid, trichloroacetic acid, etc. After centrifugation, the supernatant is collected and aliquots are injected or further extracted by an extraction method. A significant amount of the proteins present is precipitated in the presence of the organic solvent. However, a large fraction of the proteins remains soluble and can cause interference with the analytes. In addition, coprecipitation of the analyte with the proteins may occur, while other low molecular weight compounds such as lipids stay in the sample (2).

In most analyses, the analyte has to be isolated from the sample by using an extraction technique. Until recently, exclusively liquid–liquid extraction (LLE) was applied. The analyte is extracted from the sample matrix with an organic solvent with a large affinity for the analyte. The disadvantages of this technique are well known: The use of large volumes of extraction solvent, the formation of emulsions during the mixing procedure, time consumption during evaporation procedures, and coextraction of proteins and other matrix components (3,4).

Solid phase extraction (SPE) is an extraction process that utilizes a solid and a liquid phase. This technique tends to replace the classical liquid–liquid extraction. The principle of SPE is similar to that of LLE, involving a partitioning of compounds between two phases. Disposable minicolumns packed with different particle size sorbents with either non-polar or polar functional groups are used. The sorbents are modified silica, unmodified silica, alumina, polymers etc. SPE consists of four steps: 1. Activation of the sorbent functional groups by solvation in order to be prepared to interact with the sample. 2. Sample loading and analyte retention. 3. Washing with an appropriate solvent to remove undesired matrix components. 4. Elution of analytes of interest. The extract is directly injected or evaporated and reconstituted in the mobile phase, possibly after addition of internal standard. Optimization assays can be performed in each of these steps. Gravity, positive force, vacuum, or centrifugation can be used for SPE procedures (3,5).

Introduction of the new generation of hydrophilic–lipophilic polymers, such as OASIS by WATERS and Abselut by Varian, has simplified the extraction protocol and, thus, reduced the time needed for sample preparation. These are designed to extract an extensive spectrum of analytes: acidic, basic, and neutral,

whether polar or non-polar, with no conditioning required, as they are water wettable. The so called hydrophilic-lipophilic balanced sorbent is a co-polymer of divinylbenzene and *N*-vinylpyrolidone. The pyrolidone group in the polymer is a hydrogen acceptor. Compared to reversed phase silica phases, copolymeric sorbents have a higher binding capacity, are water wettable, are stable over an extended pH range (0–14), and have predictable mechanisms of interactions with residual silanols (5,6).

The latest advancement in SPE devices is the disc format, such as the Solid Phase Extraction Concentrator (SPEC) microcolumn. Several types of disk extraction media are commercially available. The most prevalent are glass fiber-based products and PTFE-based products. Glass fiber-based perform as depth filters, which are fibrous and permeable materials with the advantage over surface filters (PTFE based) of increased resistance to clogging when processing particle-laden samples. SPEC discs consist of a glass fiber supporting matrix in which silica and bonded silica particles are embedded. The resulting rigid disc can be considered as an array of interconnected closely packed porous particles of small diameter. Their advantages compared to conventional packed-bed are: high reproducibility (lot-to-lot and within lot), selective analyte retention, minimal channeling, cleanliness of sample without interferences, more efficient processing with fewer steps, faster throughput, and reduced solvent usage. The typical extraction sequence is similar to the traditional SPE, though significantly lower volumes of organic solvents are required. Disc conditioning involves no more than 500 μL of an organic solvent, followed by the same volume of solvent exchange to match the sample matrix. After washing off the interferences by using an appropriate solvent in which the analyte is insoluble, analytes are eluted by using the solvent that disrupts the interaction of analyte with the bonded phase (7–10).

Analgesics containing paracetamol are widely used since aspirin is no longer recommended as an analgesic or antipyretic for children because of reported associations with Reye's syndrome (11). The use of paracetamol alone, or in combination with other drugs, such as caffeine and codeine as analgesic and antipyretic is well established in pharmaceutical formulations. Additionally, it is used as a probe to measure conjugate (Phase II) metabolism. Studies that examine the pharmacokinetics of a drug substance often require frequent blood sampling to adequately describe the disposition characteristics. This is not usually a problem with adults, however, frequent blood sampling in children may cause the removal of excessive amounts of blood, thus, altering hemodynamics or affecting the study outcome. Therefore, assays using a minimal amount of blood sample offer a significant advantage. Quantitative determination of active ingredients in pharmaceutical preparations is reported using various techniques such as spectrophotometry and chromatography. Among the latter, HPLC constitutes the most popular (12–21).

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The determination of active ingredients of analgesics in biological fluids (overdose monitoring) and in pharmaceutical dosage forms (quality control) remains of great interest. There are no available methods in the literature concerning the simultaneous determination of paracetamol, caffeine, and codeine in biological fluids. No published literature exists for solid phase extraction optimization assays to obtain the highest recoveries for studied analytes.

The objective of this study was to investigate different solid phase extraction protocols with regards to analyte retention and elution as well as to interference elimination. Recovery rates were measured using different sorbent materials: conventional C_{18} and C_8 and novel bifunctional polymeric materials and different eluent solvents. Novel materials were examined with and without conditioning. C_{18} in disc format was also investigated.

An analytical method was initially developed for the simultaneous determination of the three compounds and this method was validated for its performance prior to its application to the SPE comparison study. The method was also evaluated by precision assay of commercial pharmaceuticals.

EXPERIMENTAL

Reagents and Materials

Paracetamol was purchased from Merck (Darmstadt, Germany). Caffeine was purchased from Sigma (St. Louis, MO, USA). Ethanolic solution of codeine (10,000 μg/mL) was supplied by Wyeth, Ontario, Canada). Lamotrigine, used as internal standard, was from Wellcome Foundation, LTD, London, UK). Ammonium acetate was from Merck. All compounds were of analytical purity.

HPLC grade methanol was obtained from Panreac (Barcelona, Spain), while HPLC grade isopropanol and acetonitrile were supplied from Riedel-de-Haen (AG, Seelze, Germany). De-ionised water was used throughout analysis.

Urine samples were obtained from healthy volunteers. Serum samples were kindly provided from the Blood Donation Unit of a State Hospital.

Lonarid N tablets and Lonalgal suppositories were both produced by Boehringer Ingelheim and Panadol Extra tablets produced by SmithKline Beecham were purchased from a local pharmacy.

HPLC Instrumentation

The chromatographic system operating in isocratic mode, consisted of the commercial components: a Shimadzu (Kyoto, Japan) LC-10A pump, an

SSI 500 variable UV/VIS detector (SSI, State College, PA, USA.) operating at 240 nm and a sensitivity setting of 0.002 (AUFS), a 7161 Rheodyne (California, USA) injection valve with a 20 μL loop, and a HP 3396 II integrator (Hewlett-Packard, Avondale, PA, USA). The analytical column, an Inertsil C_8, 250 mm × 4 mm ID, 5 μm, was purchased from MZ Analysentechnik (Mainz, Germany).

SPE minicolumns used in this study include: C_{18} supplied by Varian, C_{18} and C_8 supplied by Merck, C_{18}, Discovery by Supelco (Bellefonte, PA, USA), C_8 by Alltech (Deerfield, IL, USA), SPE minicolumns with hydrophilic and lipophilic properties, Oasis HLB, were purchased from Waters (Waters Corporation, Massachusetts, USA), and ABSELUT–NEXUS from VARIAN. SPEC disc format C_{18} were a gift from SPEC, A Division of Ansys Diagnostics (Lake Forest, CA, USA).

A Vac-Elut vacuum manifold column processor used for SPE was purchased from Analytichem International, a division of Varian (Harbor City, USA). All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

UV spectra for selecting the monitoring wavelength of detection were provided by a Varian DMS 100S UV/Vis double-beam spectrophotometer prior to chromatographic method development.

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of mobile phase, using 0.2 μm membrane filters, obtained from Schleicher and Schuell (Dassel, Germany).

Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) or by vigorous Helium sparging prior to use.

A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pre-treatment.

Preparation of Standard Solutions

Individual aqueous stock solutions (100 ng/μL) were prepared for paracetamol and caffeine and the internal standard. Initial ethanolic solution of codeine (10,000 ng/μL) was further diluted with water to give the stock of 100 ng/μL. Mixtures of the three components were prepared by appropriate dilutions containing the internal standard. Quantitation was based on linear regression analysis of analyte to internal standard peak area ratio versus analyte concentration in ng/μL. All samples were stored refrigerated at 4°C. These solutions were found to be stable throughout experimental analyses.

Chromatographic Conditions

The analytical column, Inertsil C_8, 250 mm × 4 mm ID, 5 μm, was used at ambient temperature 22°C. Various mobile phases were tested with different methanol content. The final eluting system was selected in terms of optimum resolution and peak shapes of investigated compounds with a short retention time. The elution system was comprised of two phases: phase A: 0.05 M ammonium acetate and phase B: CH_3OH at a volume ratio 40:60. This was delivered isocratically at a flow rate of 0.93 mL/min. Inlet pressure observed was 235–240 kg/cm^2. Eluent was monitored at 240 nm. The sensitivity setting of the UV–VIS detector was adjusted at 0.002 AUFS.

A wide variety of compounds, such as xanthine derivatives, quinine, and bamifylline were assayed towards their application as internal standard. Lamotrigine was selected due to its elution properties, spectra characteristics, and its absence in biological fluids. The concentration of internal standard was 1 ng/μL.

RESULTS AND DISCUSSION

Calibration Data and Sensitivity

Calibration of the method was performed by injection of standards, covering the range 0.001–40.0 ng/μL. Each sample was injected six times. Linear correlation between absolute injected amount or concentration and peak area ratio, with lamotrigine, as internal standard at a concentration of 1.0 ng/μL, was observed.

The results of the statistical treatment of calibration data are summarised in Table 1. The sample analysis time of analgesics, in the proposed method, is less than 9 min as shown in Fig. 1, that illustrates a typical chromatogram of separated analytes.

The minimum detectable concentration LOD was defined as a peak height that produces three times baseline noise at 0.0005 AUFS. This was found to be 0.02 ng for paracetamol, 0.04 ng for caffeine, and 0.1 ng for codeine. The LOQ

Table 1. Linear Ranges, Correlation Coefficients and Limits of Detection for the Three Analgesic Pharmaceutical Constituents, in the Presence of Lamotrigine (Internal Standard)

Analytes	Linear Range (ng/μL)	Correlation Coefficient	Limit of Detection (ng)
Paracetamol	0.003–6	0.997	0.02
Caffeine	0.008–8	0.994	0.04
Codeine	0.02–40	0.997	0.1

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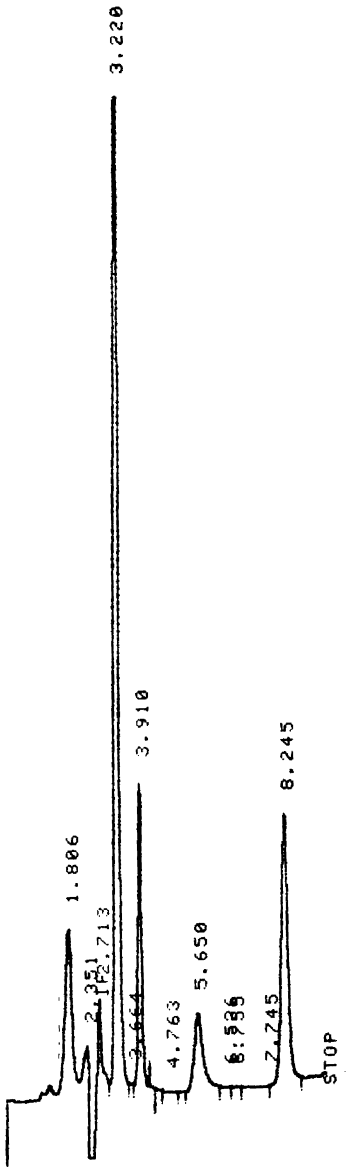

Figure 1. High performance liquid chromatogram of paracetamol 3.220 min, caffeine 3.910 min, codeine 5.650 min, with 1 ng/μL lamotrigine 8.245 min, as internal standard. Chromatographic conditions are described in text.

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was the lowest concentration of calibration standards with acceptable precision and accuracy, and was found to be 0.06 ng for paracetamol, 0.15 ng for caffeine, and 0.35 ng for codeine. The upper limit of the calibration curve was 6 ng/μL for paracetamol, 8 ng/μL for caffeine, and 40 ng/μL for codeine.

Method Validation: Accuracy, Precision, Stability

The precision of the method based on within-day repeatability was performed by replicate injections ($n = 8$) of three standard solutions covering different concentration levels: low, medium, and high. Statistical evaluation revealed relative standard deviations at different values. Results are shown in Table 2.

The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days. Reproducibility results are illustrated in Table 2.

Accuracy was determined by replicate analysis of three different levels (0.5, 3.0, and 5.0 ng/μL) and calculating the recoveries of actual versus spiked values.

Table 2. Within-Day ($n = 8$) and Day-to-Day Precision and Accuracy Study (Three Measurements over a Period of 8 Consecutive Days) for Determination of Analgesics Constituents

	Within-Day			Day-to-Day		
Added (ng)	Found ± SD (ng)	RSD	R[a] (%)	Found ± SD (ng)	RSD	R[a] (%)
Paracetamol						
10	10.4 ± 0.6	5.8	104.0	10.6 ± 0.3	2.8	106.0
60	58.8 ± 1.9	3.2	98.0	63.2 ± 4.2	6.6	105.3
100	98.6 ± 3.1	3.1	98.6	98.8 ± 3.5	3.5	98.8
Caffeine						
10	9.9 ± 0.3	3.0	99.0	9.7 ± 0.3	3.1	98.0
60	60.8 ± 1.5	2.5	101.3	63.1 ± 2.8	4.4	105.2
100	101.1 ± 2.2	2.0	101.1	105.0 ± 4.9	4.7	105.0
Codeine						
10	10.3 ± 0.5	4.9	103.0	10.3 ± 0.6	5.8	103.0
60	60.5 ± 1.6	2.6	100.8	57.7 ± 2.2	3.8	96.2
100	97.2 ± 3.5	3.6	97.2	97.8 ± 2.5	2.6	97.8

[a]R = recovery.

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The stability of solutions was verified by storing sample solutions, refrigerated for one month. Concentrations were measured periodically (one, two, three, and four weeks).

Application to Pharmaceuticals

Three to five tablets were weighed and the mean value was recorded. After finely powdering the tablets in a porcelain mortar, a solution of the analgesic tablet was prepared by dissolving the appropriate amount that corresponds to the average weight of one tablet in 1000 mL water. The insoluble excipients were removed by filtration, and the filtrate diluted to yield three different concentrations within the working range of the analytes containing 1 ng/μL of lamotrigine. Due to the high difference in the analyte concentration levels in pharmaceuticals, six dilute solutions were eventually necessary.

Five suppositories were weighed to record the average weight and then cut into small pieces. Portions of each, corresponding to the mean weight, were placed into a glass beaker and incubated at 38°C for 15 min. Paracetamol and codeine were extracted by 15 mL methanol in an ultrasonic bath, and the supernatant solution was transferred to a 100 mL volumetric flask and diluted to volume with water. Dilute solutions were prepared at three concentrations for each analyte within the working range. All solutions contained lamotrigine at 1 ng/μL. Once again, because of the high differences in the initial concentration of paracetamol and codeine in the pharmaceutical, six solutions were prepared to cover the working range for each analyte. Table 3 contains the accuracy data of analgesic pharmaceuticals. Fig. 2 illustrates a typical chromatogram of an analgesic pharmaceutical preparation analysed for its active ingredients.

Solid Phase Extraction Protocols

In order to investigate the recovery efficiency of several SPE sorbents from different manufacturers and in different formats, the following extraction protocols were established for further optimization. The studied adsorbents were: RP-18 (Merck, Discovery by Supelco, Varian), RP-8 (Alltech, Adsorbex by Merck), hydrophilic-lipophilic (Oasis by Waters and Nexus by Varian), and Disc-format C_{18} by SPEC, Ansys.

Recovery was calculated by comparing the peak area ratio of the analytes to the internal standard, from the processed sample to the corresponding peak area

Table 3. Results of Commercial Analgesic Mixtures Analysis

	Labeled Amount	*Found ± SD	RSD	R (%)
Panadol-extra				
Paracetamol	500 mg[a]	496 ± 17	3.4	99.2
Caffeine	65 mg[a]	62 ± 2	3.3	95.4
Lonarid-N				
Paracetamol	400 mg[a]	430 ± 8	1.8	107.5
Caffeine	50 mg[a]	50 ± 2	4.1	100.0
Codeine	10 mg[a]	10 ± 0.8	8.0	100.0
Lonalgal				
Paracetamol	1000 mg[b]	1011 ± 20	2.0	101.1
Codeine	30 mg[b]	31 ± 2	6.4	103.3

*mg (Mean value of six measurements at three concentration levels $n = 18$).
[a]Per tablet.
[b]Per suppository.

ratio of the non-processed sample. Table 4 illustrates the examined protocols for each sorbent.

The protocols under assay gave various recovery results, which are tabulated in Table 5. As can be seen, isopropanol was the best eluting solvent for paracetamol and codeine, while OASIS was the best sorbent for the three compounds. Acetonitrile was the best eluting solvent for the three compounds when OASIS was used as sorbent; though conditioning was unavoidable as shown in Figs. 3b and 3c. Methanol was shown to be the best solvent to elute the three analytes from the non-conditioned NEXUS sorbent, whereas activation of the sorbent improved performance, especially when acetonitrile and isopropanol were used as eluent. Fig. 3a illustrates the chromatogram obtained after SPE using NEXUS without conditioning, and Figs. 3b and 3c the chromatograms using OASIS with and without conditioning, respectively. It is obvious that conditioning of OASIS sorbent is more than necessary.

The disc format of C_{18} sorbent gave moderate recoveries, while low volumes of solvents were consumed.

The five protocols that gave the highest recovery rates for standard solutions are separately shown in Table 6. These were selected for further investigation when real samples blood serum and urine were to be analyzed.

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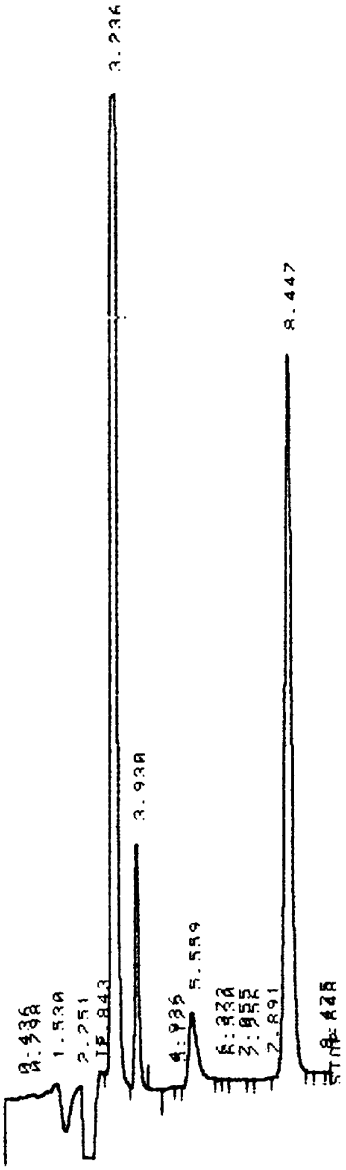

Figure 2. High performance liquid chromatogram of determination of Paracetamol 3.236 min, Caffeine 3.930 min, Codeine 5.559 min, in pharmaceutical preparation (tablet) with internal standard 1 ng/μL Lamotrigine 8.447 min.

Table 4. Setup Parameters for Solid Phase Extraction Optimization

Protocols	A	B	C	D[a]	E[b]
Conditioning	2 mL CH_3OH 2 mL H_2O	2 mL CH_3OH 2 mL H_2O	2 mL CH_3OH 2 mL H_2O	No conditioning	500 µL CH_3OH 500 µL H_2O
Sample addition	200 µL standard solution: 0.5–1.0 and 3.0 ng/µL				100 µL standard solution
Washing	2 mL H_2O				500 µL H_2O
Drying	The sorbent is dried under vacuum				
Elution	2 mL CH_3OH	2 mL CH_3CN	2 mL $CH_3CH(OH)CH_3$	2 mL $CH_3OH/CH_3CN/CH_3CH(OH)CH_3$	1 mL $CH_3OH/CH_3CN/CH_3CH(OH)CH_3$
Evaporation	To dryness under nitrogen 45°C				
Reconstitution	To 200 µL internal standard solution				

[a]Only applicable to OASIS and NEXUS.
[b]Applicable to Disc Format C-18.

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Table 5. Recovery Results % of Analgesic Compounds Using the Examined SPE Protocols (Mean Values of Six Measurements at Three Concentration Levels)

	Paracetamol R % ± SD			Caffeine R % ± SD			Codeine R % ± SD		
	CH_3OH	CH_3CN	2-Propanol	CH_3OH	CH_3CN	2-Propanol	CH_3OH	CH_3CN	2-Propanol
Varian C_{18}	85.8 ± 0.5	79.5 ± 0.8	**89.9**[a] ± 4.0	**103.3** ± 5.3	51.5 ± 1.5	91.0 ± 2.7	40.1 ± 1.3	71.8 ± 2.2	**87.7** ± 5.2
Ansys-Disc C_{18}	67.1 ± 0.7	69.1 ± 3.4	**72.2** ± 2.6	**84.1** ± 0.6	65.1 ± 0.7	72.4 ± 3.0	65.9 ± 2.2	60.0 ± 1.3	**84.0** ± 3.5
Supelco C_{18}	64.3 ± 2.3	63.4 ± 1.2	**86.2** ± 0.4	**81.3** ± 2.0	50.3 ± 1.3	72.8 ± 2.1	42.6 ± 1.3	72.6 ± 4.0	**84.8** ± 3.4
Merck C_{18}	62.4 ± 4.1	**79.8** ± 0.1	68.0 ± 1.4	74.2 ± 3.5	**84.9** ± 1.9	58.3 ± 4.3	**89.8** ± 3.0	55.1 ± 0.7	76.4 ± 1.1
Alltech C_8	**76.3** ± 1.0	62.0 ± 1.6	67.9 ± 1.0	**86.9** ± 0.2	45.9 ± 1.9	**86.5** ± 0.3	44.9 ± 0.3	64.7 ± 2.3	**65.1** ± 0.1
Merck C_8	**85.6** ± 4.0	59.6 ± 3.1	58.4 ± 2.4	58.2 ± 2.4	**73.1** ± **1.7**	**74.4** ± 1.8	**76.1** ± 5.6	50.1 ± 0.7	66.8 ± 1.5
OASIS	54.7 ± 3.4	**104.6** ± 4.2	29.3 ± 0.6	57.8 ± 2.5	**102.7** ± 3.6	66.04 ± 3.6	44.1 ± 3.0	**100.3** ± 6.8	40.5 ± 2.8
NEXUS	80.2 ± 5.7	**86.4** ± 0.3	**85.6** ± 1.0	**73.2** ± 3.8	67.3 ± 4.8	58.7 ± 2.9	40.6 ± 0.6	**74.8** ± 1.0	70.8 ± 3.8
NEXUS, nc	**101.6** ± 6.4	79.0 ± 3.2	72.8 ± 4.1	**92.9** ± 2.6	75.6 ± 2.0	60.1 ± 0.2	**79.0** ± 1.3	72.6 ± 1.3	59.1 ± 1.4

nc = Non conditioned.

[a]Higher percentage recovery yielded from different eluent solvent, for each analyte is bold typed.

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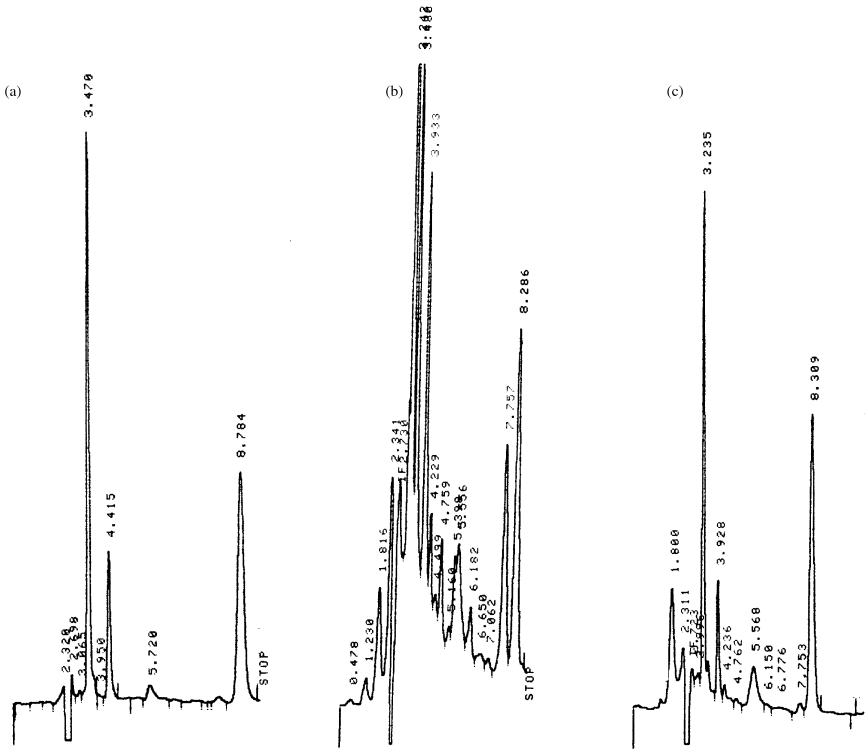

Figure 3. (a) High performance liquid chromatogram of determination of Paracetamol 3.470 min, Caffeine 4.415 min Codeine 5.720 min, with internal standard 1 ng/μL Lamotrigine 8.784 min after SPE on non-conditioned NEXUS. (b). High performance liquid chromatogram of determination of Paracetamol 3.480 min, Caffeine 3.933 min, Codeine 5.556 min, with internal standard 1 ng/μL Lamotrigine 8.309 min. after SPE on non- conditioned OASIS. (c). High performance liquid chromatogram of determination of Paracetamol 3.235 min, Caffeine 3.928 min, Codeine 5.568 min, with internal standard 1 ng/μL Lamotrigine 8.309 min after SPE on preconditioned OASIS.

Application to Biological Fluids: Blood Serum-Urine

Human Blood Serum

Aliquots of 40 μL pooled human blood serum were spiked with 200 μL of standard solution, at concentration levels of 0.5, 1.0, and 3.0 ng/μL. Each sample was treated with 200 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was centrifuged at 3500 rpm for 10 min and the supernatant was diluted with 1.5 mL water. Subsequently, the sample was

Table 6. Five Protocols Providing Higher Recovery Rates for Standard Solutions of Analgesic Compounds

SPE Sorbent	Elution Solvent	Paracetamol R % ± SD[a]	Caffeine R % ± SD[a]	Codeine R % ± SD[a]
C_{18}-Varian	isopropanol	89.9 ± 4.0	91.0 ± 2.7	87.7 ± 5.2
Supelco-Discovery C_{18}	isopropanol	86.2 ± 0.4	72.8 ± 2.1	84.8 ± 3.4
Ansys Disc format C_{18}	isopropanol	72.2 ± 2.6	72.4 ± 3.0	84.0 ± 3.5
OASIS (cond.)	CH_3CN	104.6 ± 4.2	102.7 ± 3.6	100.3 ± 6.8
NEXUS Varian n.c.	CH_3OH	101.6 ± 2.0	92.9 ± 2.6	79.0 ± 1.3

[a]Mean value of six measurements at three concentration levels.

slowly applied to the solid phase cartridge and treated according to the five protocols that gave the highest recovery rates, as shown in Table 6. After elution with the appropriate solvent, each sample was evaporated to dryness under a gentle stream of nitrogen. Reconstitution to 200 μL was performed with 1 ng/μL internal standard solution. No interference from endogenous compounds was observed. Absolute recovery was measured at the three concentration levels and calculated by comparing the concentrations for spiked serum samples with those for direct injection of pure compounds. The absolute recoveries are shown in Table 7.

Table 7. Absolute Recovery of Analgesic Compounds from Human Blood Serum and Urine

SPE Sorbent	Elution Solvent	Paracetamol R % ± SD[a]	Caffeine R % ± SD[a]	Codeine R % ± SD[a]
Serum				
C_{18}-Varian	isopropanol	98.8 ± 7.0	98.4 ± 3.1	90.1 ± 0.5
Supelco-Discovery C_{18}	isopropanol	88.1 ± 3.1	96.6 ± 2.0	93.0 ± 2.6
Disc format C_{18}	isopropanol	76.4 ± 1.7	90.0 ± 0.6	86.9 ± 0.7
OASIS (cond.)	CH_3CN	95.5 ± 1.3	99.0 ± 1.7	99.4 ± 0.4
NEXUS Varian n.c.	CH_3OH	86.2 ± 3.1	99.8 ± 3.8	84.3 ± 3.0
Urine				
C_{18}-Varian	isopropanol	79.5 ± 0.6	82.0 ± 3.2	91.1 ± 0.6
Supelco-Discovery C_{18}	isopropanol	84.4 ± 1.1	79.3 ± 0.3	94.0 ± 1.5
Disc format C_{18}	isopropanol	93.8 ± 1.7	67.8 ± 1.0	73.8 ± 0.4
OASIS (cond.)	CH_3CN	94.2 ± 1.8	97.1 ± 1.9	93.2 ± 0.1
NEXUS Varian n.c.	CH_3OH	84.5 ± 2.8	90.5 ± 2.4	83.3 ± 1.3

[a]Mean value of six measurements at three concentration levels.

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A high performance liquid chromatogram of analgesic compounds extracted from human blood serum is shown in Fig. 4a.

Urine

Aliquots of 100 μL of pooled urine sample were spiked with 200 μL of standard solution at concentration levels of 0.5, 1.0, and 3.0 ng/μL. Each sample was treated with 200 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was centrifuged at 3500 rpm for 10 min and the supernatant was diluted with 1.5 mL water. Subsequently, the sample was slowly applied to the solid phase cartridge and extracted according to the five selected protocols. After elution with the appropriate solvent, each sample was evaporated to dryness under a gentle stream of nitrogen. Reconstitution to 200 μL was performed with a 1 ng/μL internal standard solution. Absolute recovery was measured at the three concentration levels and calculated by comparing the concentrations for spiked urine samples with those for direct injection of pure compounds. The absolute recoveries are shown in Table 7. No interference from matrix compounds was observed.

A high performance liquid chromatogram of analgesic compounds extracted from human urine is shown in Fig. 4b.

CONCLUSIONS

Paracetamol (Acetaminophen or *N*-acetyl-*p*-aminophenol) was chosen for this assay of its isolation from biological fluids, as it is a widely used analgesic and antipyretic agent available alone or in combination with other drugs, such as codeine and caffeine, in multi-component analgesic mixtures.

The ideal sample pretreatment technique should be simple and rapid because it takes almost 60% of the total analysis time. It should also provide high extraction efficiency with quantitative and reproducible analyte recoveries; be specific for the analytes, leaving interference behind; provide high sample throughput using as few manipulation steps as possible so that analyte losses are eliminated; use the minimum solvent amount compatible with many analytical techniques; be of low cost; be friendly to the environment; and meet all requirements in terms of sensitivity and clean up.

The methods used for sample preparation in bioanalysis samples prior to their introduction into the chromatographic system, include extraction or direct injection after protein precipitation. The latter, though, being rapid and simple leaves behind unwanted materials. Solid Phase Extraction sufficiently eliminates sample matrix and provides high percentage recoveries when the proper

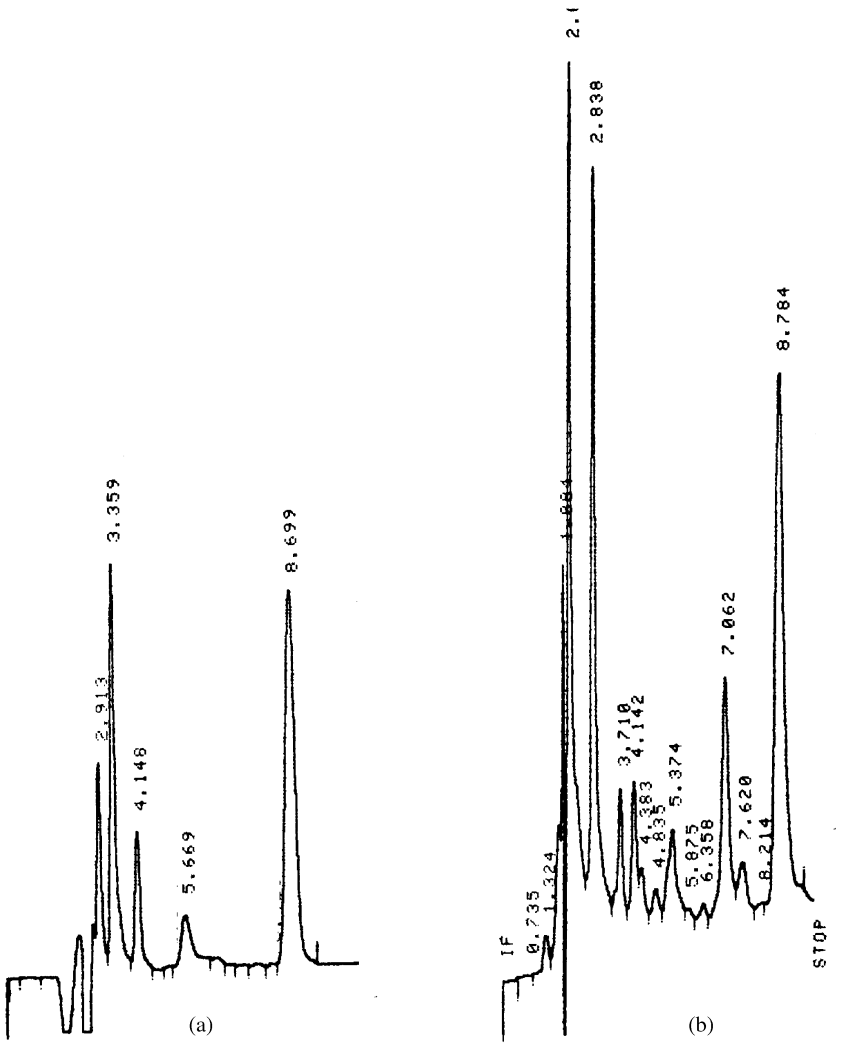

Figure 4. (a) High performance liquid chromatogram of determination of Paracetamol 3.359 min, Caffeine 4.148 min, Codeine 5.669 min, in spiked human blood serum samples with internal standard 1 ng/μL Lamotrigine 8.699 min, after SPE using preconditioned OASIS. (b) High performance liquid chromatogram of determination of Paracetamol 3.710 min, Caffeine 4.142 min, Codeine 5.374 min in blank urine sample with internal standard 1 ng/μL Lamotrigine 8.784 min after SPE using preconditioned OASIS.

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procedure protocol is selected. This can be achieved by performing optimization of retention and elution conditions.

The analytical method developed in this study provides high accuracy and precision with satisfactory repeatability, low limits of detection, and high recoveries from pharmaceutical formulations.

Several SPE protocols were studied in order to attain the optimal extraction conditions of analgesic compounds from biological fluids. New commercially available sorbents, which have been presented as universal extraction sorbents capable of extracting hydrophilic, lipophilic, acidic, basic, and neutral compounds, were studied with and without sorbent conditioning, however, this was found to be unavoidable in the case of OASIS sorbent. Each of the examined sorbents could provide satisfactory recovery rates by selecting the appropriate eluent for individual compounds. However, preconditioned OASIS, with acetonitrile as eluent, provided the most satisfactory results for the three analytes and for both biological matrices. C_{18} sorbent from Varian and Supelco gave equivalent recovery with isopropanol as eluent, though only in the case of serum sample. Non-conditioned Nexus by Varian gave high recovery for caffeine using methanol as eluent. C_{18} sorbent in disc format gave satisfactory recoveries with the advantage of low solvent consumption. Small volumes of biological fluids were required: 40 mL of blood serum and 100 mL of urine.

REFERENCES

1. Lord, H.; Pawliszyn, J. J. Chromatogr. A. **2000**, *902* (1), 17–63.
2. Blanchard, J. J. Chromatogr. **1981**, *226*, 455–460.
3. Papadoyannis, I.N. Sample Preparation Prior to HPLC Analysis, Solid Phase Extraction Techniques. In *International Conference on Instrumental Methods of Analysis, Modern Trends and Applications*, Chalkidiki, Greece, 19–22 September 1999, 443–451; Ochsenkün-Petropulu, M., Ochsenkün, K.M., Eds., I. Katsarakis: Athens, 1999.
4. Majors, R. LC-GC. **1997**, *2*, 93–101.
5. Hennion, M.-C. J. Chromatogr. A. **1999**, *856* (1–2), 3–54.
6. Bouvier, E.; Martin, D.M.; Iraneta, P.; Capparella, M.; Cheng, Y-F.; Philips, D. LC–GC, **1997**, *9,* 577–583.
7. Henry, M.P. *SPEC Reproducibility Monograph*; Ansys Inc., 1994.
8. Blevins, D.D.; Henry, M.P.; O' Donnel, C.M. *Advantages of SPEC Disc Extractions over Conventional Packed Bed SPE*; SPEC Ansys Inc., 1994.
9. *SPE Disc Products Catalog*; SPEC Ansys, 1997.
10. Blevins, D.D.; Hall, D.O. LC–GC (USA) **1998**, *5*, S16–S21.
11. Al-Obaidy, S.S.; Wan Po, A.L.; McKiernan, P.J.; Glasgow, J.F.T.; Millership, J. J. Pharm. Biomed. Anal. **1995**, *13* (8), 1033–1039.

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270 Madison Avenue, New York, New York 10016

12. Cockaerts, P.; Roets, E.; Hoogmartens, J. J. Pharm. Biomed. Anal. **1986**, *4*, 367–376.
13. El-Kommos, M.; Emara, K. Talanta **1989**, *6*, 678–679.
14. Williams, J.P.; West, K.J.; Erickson, K.I. J. Chem. Education **1992**, *69* (8), 669–670.
15. Studenberg, S.D.; Brouwer, K.L.R. Biochem. Pharmacol. **1993**, *46*, 739–748.
16. Thomas, B.R.; Fang, X.G.; Shen, P.; Ghodbane, S. J. Pharm. Biomed. Anal. **1994**, *12*, 85–90.
17. Indrayanto, G.; Sunarto, A.; Adriani, Y. J. Pharm. Biomed. Anal. **1995**, *13* (12), 1555–1559.
18. Di Pietra, A.M.; Gatti, R.; Andrisano, V.; Cavrini, V. J. Chromatogr. A. **1996**, *729* (1–2), 355–361.
19. Dinc, E. J. Pharm. Biomed. Anal. **1999**, *21* (4), 723–730.
20. Brunner, L.J.; Bai, S. J. Chromatogr. B. **1999**, *732* (2), 323–329.
21. Spell, J.C.; Stewart, J.T. J. Liquid Chromatogr. & Rel. Technol. **1999**, *22* (2), 297–305.

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